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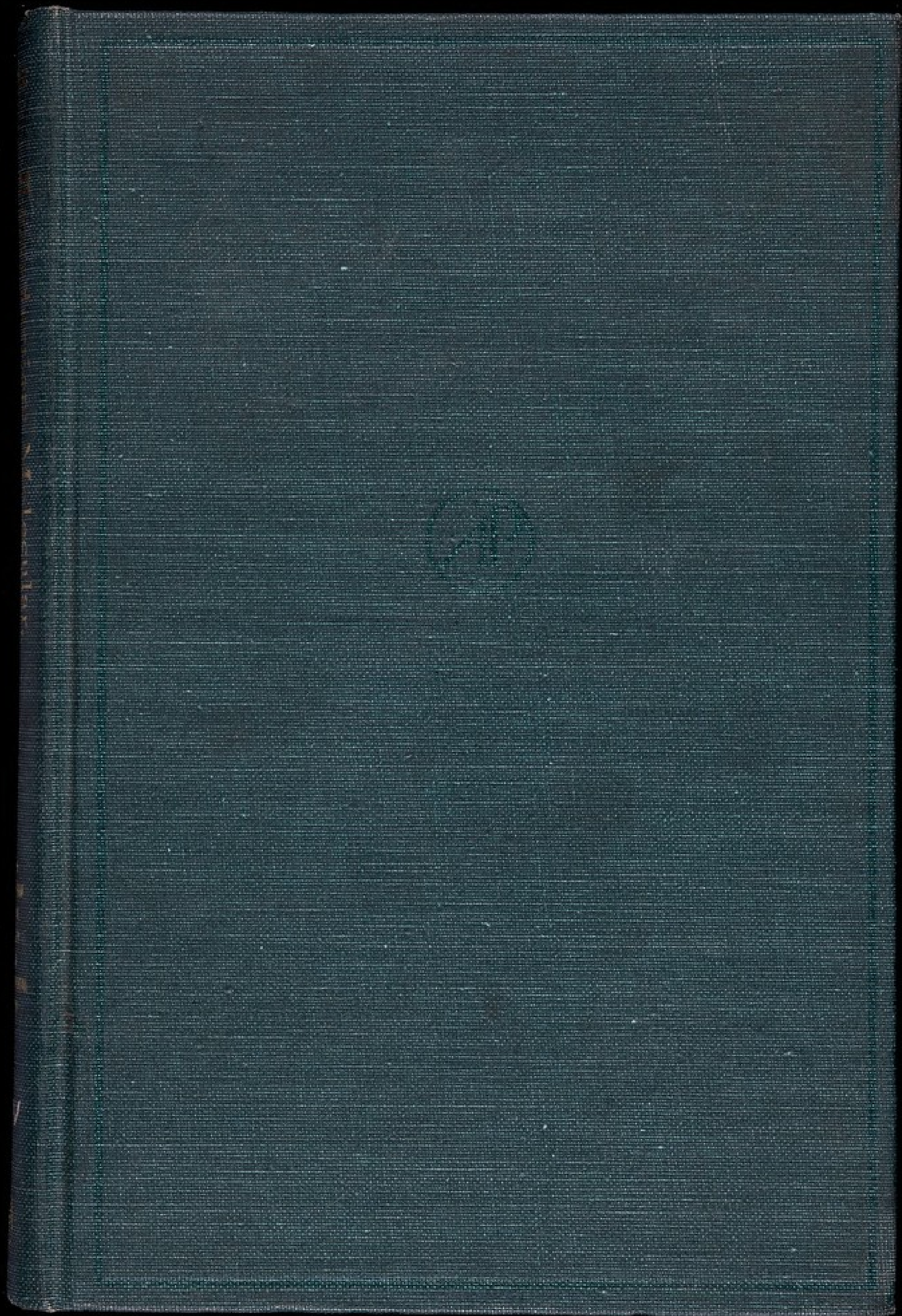

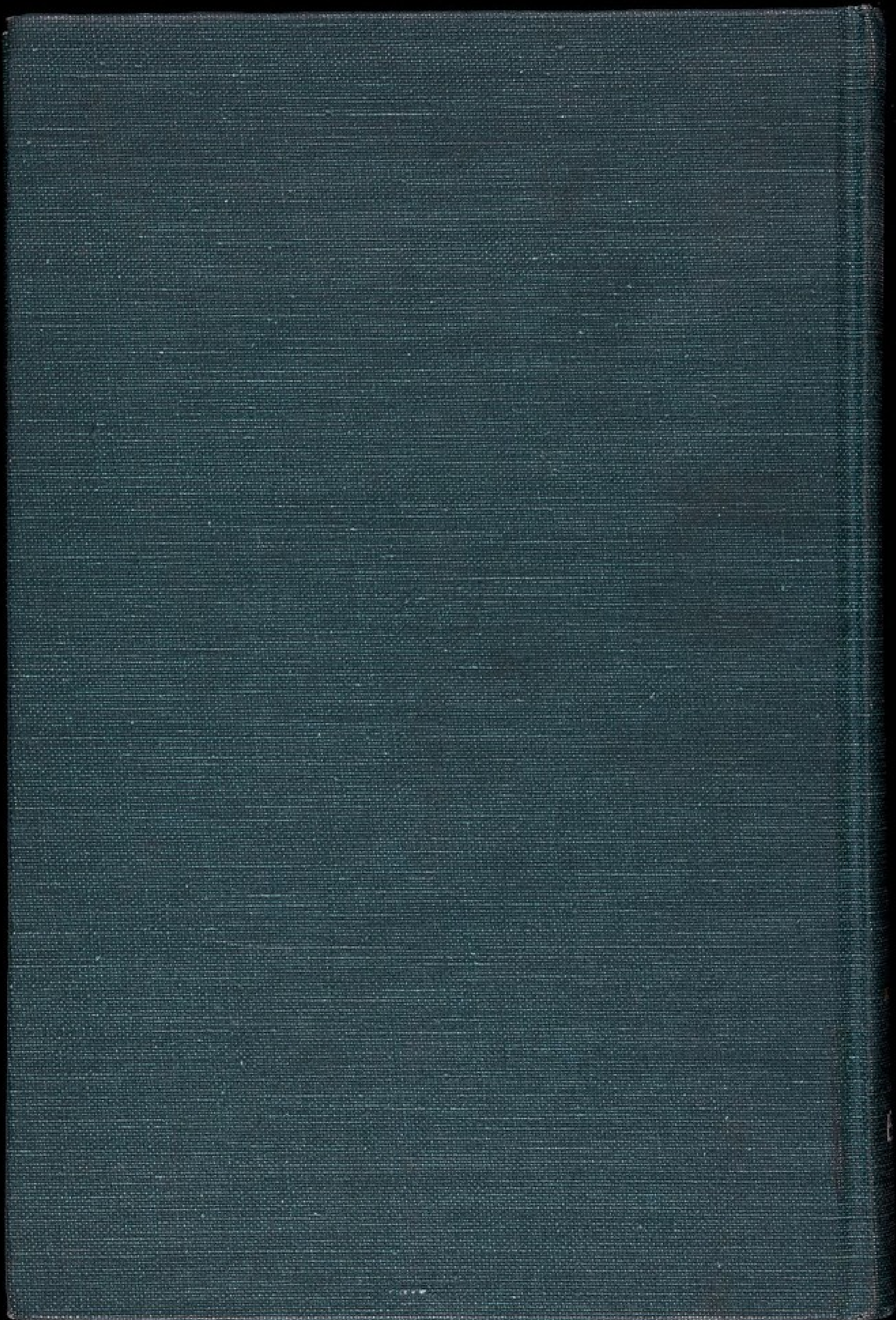

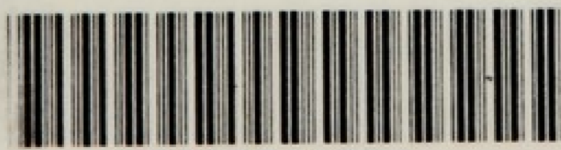
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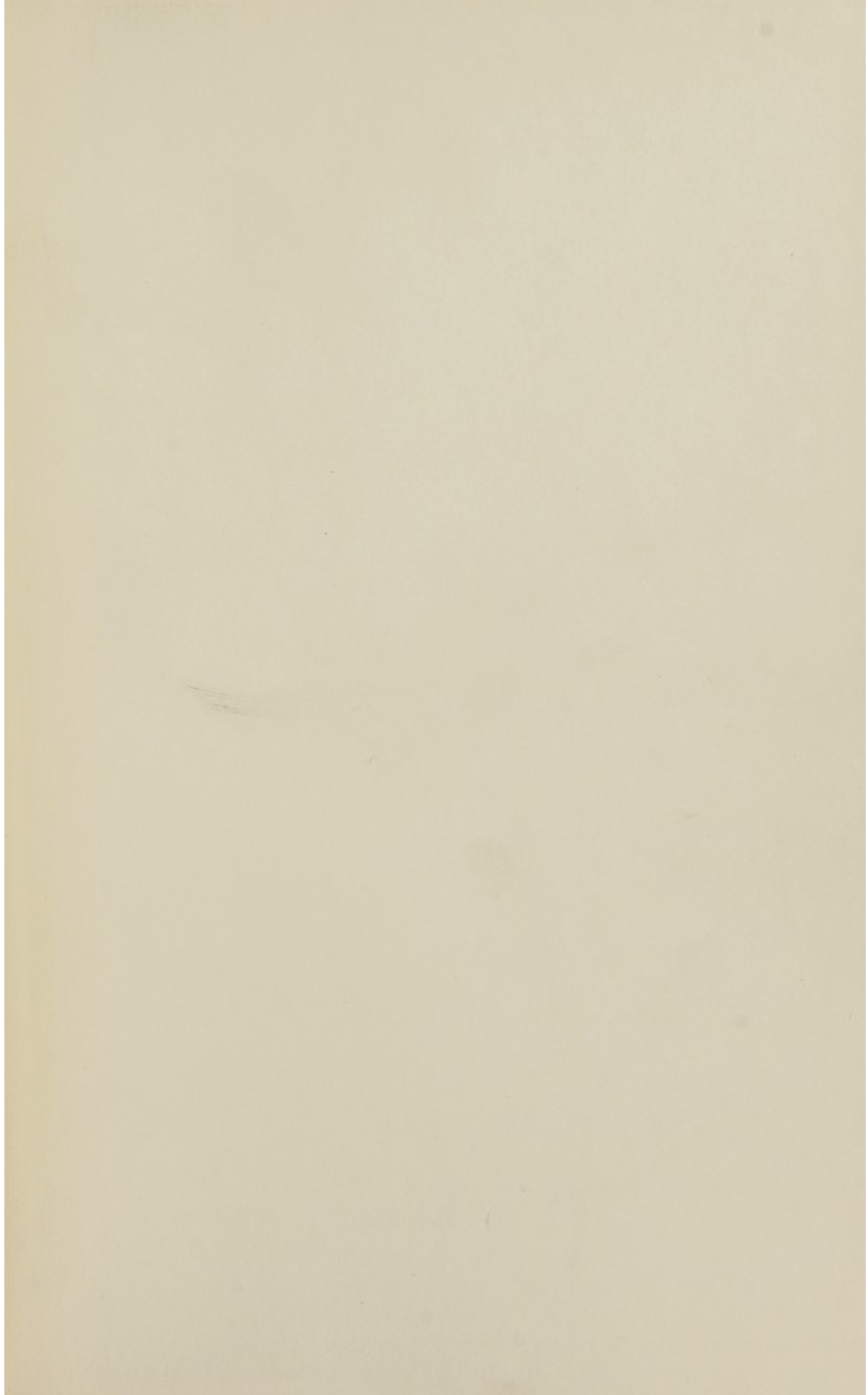

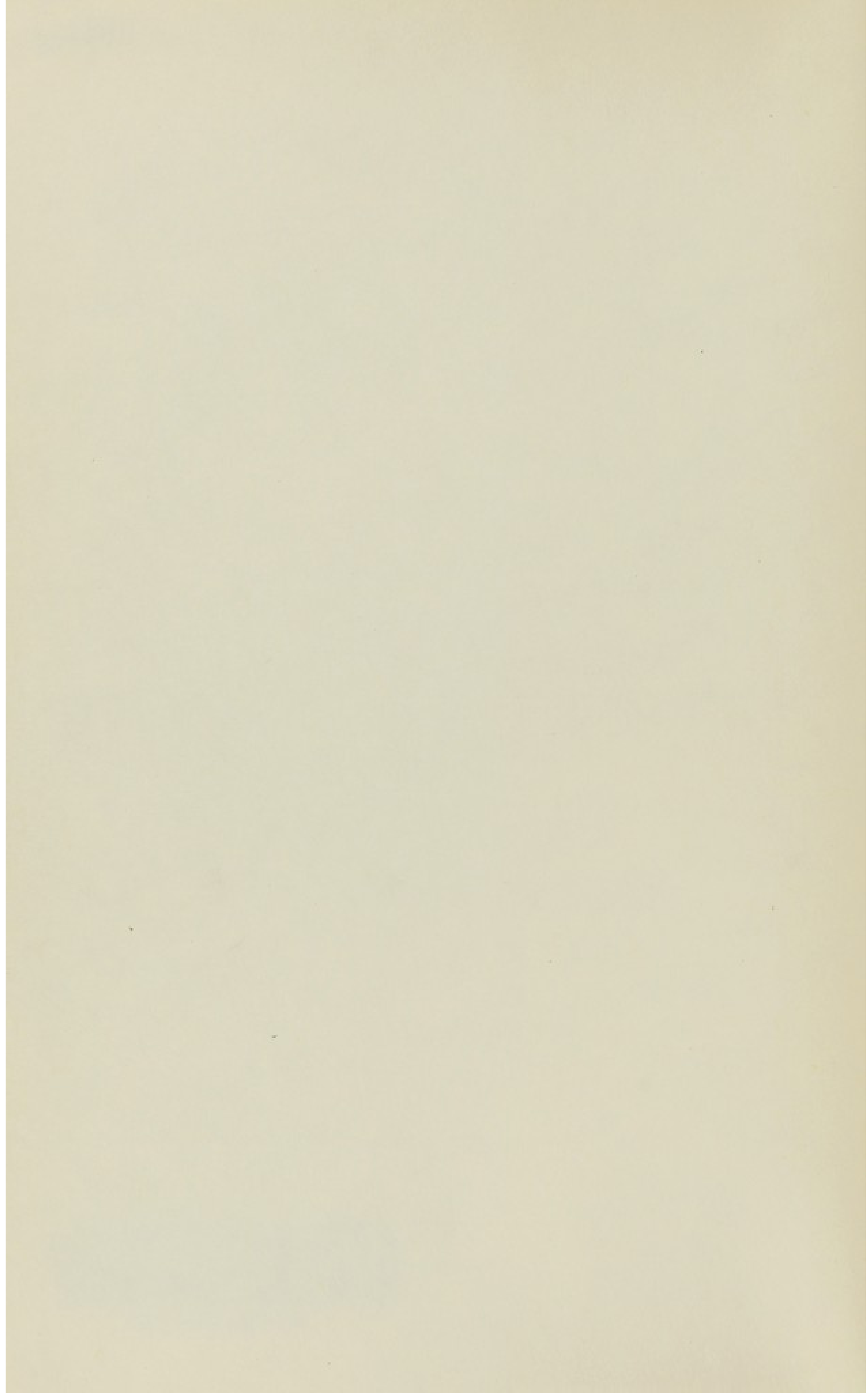

Reproduction: Molecular, Subcellular, and Cellular

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The Society for
Developmental Biology

(Formerly The Society for the Study of Development and Growth)

Carleton, Minnesota, June 1965

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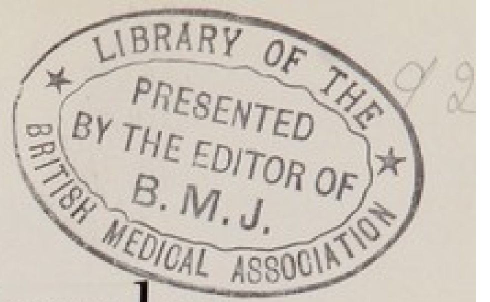

Reproduction: Molecular, Subcellular, and Cellular

Edited by

Michael Locke

Developmental Biology Center
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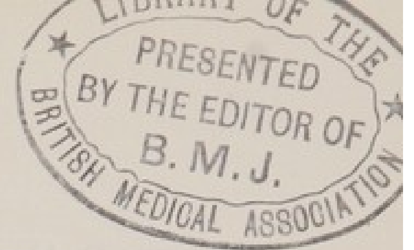
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Contents

CONTRIBUTORS AND PRESIDING CHAIRMEN v

Reproduction: Molecular, Subcellular, and Cellular

HERBERT STERN

Introduction 1
Regulation of Reproductive Mechanisms 7

REPRODUCTION AT THE MOLECULAR LEVEL

Transcription and Translation of Genes

K. C. ATWOOD

ur-Genes and the Gene Action System 17
References 32

Structural Basis of the Specificity of Antibodies

ALFRED NISONOFF AND F. P. INMAN

Introduction 39
Dissociation of Rabbit γG-Globulin into Half-Molecules ... 45
Spontaneous Reassociation of Half-Molecule Subunits at Neutral pH 47
Evidence for Univalence of Half-Molecules of Rabbit γG-Globulin 48
Reversible Dissociation of Fragment Fc 49
In Vitro Complementation of Polypeptide Subunits of γG-Globulins 51
Evidence for Antibody Activity in Isolated Heavy Chains .. 52
Antibody Activity of Recombined Heavy and Light Chains 53
Evidence for Direct Participation of Light Chains in the Active Site of an Antibody 57

Evidence That Differences in Specificity Are Associated with Differences in Amino Acid Sequence 57
Studies with Myeloma and Bence Jones Proteins 59
References 60

Interactions between Plant Viruses and Host Cells

K. W. Mundry

Introduction 65
Structure and Infectivity of Plant Viruses 66
Virus Mutation 72
The Genetic Code 79
The Size of Cistrons and the Concept of Minimal Infectious Genetic Information 84
Plant Virus Replication and Some of Its Cellular Events .. 86
References 91

Interaction of Viruses with the Genetic Material of the Host Cells

Renato Dulbecco

Introduction 95
Consequences of Virus Infection for the Genetic Material of Bacterial Cells 96
Consequences of Virus Infection for the Genetic Material of Animal Cells 101
References 104

REPRODUCTION OF SUBCELLULAR SYSTEMS

Chromosome Reproduction in Mitosis and Meiosis

C. P. Swanson and William J. Young

Introduction 107
Replication As a Process 108
Chromosome Reproduction 116
Consequences of Chromosome Reproduction 121
References 124

The Continuity of the Chloroplast in *Euglena*

JEROME A. SCHIFF AND H. T. EPSTEIN

Introduction—The Two Aspects of Organelle Continuity .. 131
The Developmental Aspect of Chloroplast Continuity 132
The Replicative Aspect of Chloroplast Continuity 143
Possible Control Mechanisms for Chloroplast Development and Replication 178
Speculations Concerning the Evolution of the Chloroplast 182
Conclusion 183
References 183

Extrachromosomal Heredity in Fungi

ADRIAN M. SRB

Introduction 191
Evidence for Extrachromosomal Heredity 193
The Origins of Extrachromosomal Variability 203
Phenotypic Effects 204
Interactions 205
Discussion 207
References 210

Final Remarks: "Why So Much DNA?"

S. GRANICK

Text 213

THE REPRODUCTION OF CELLS

Haploidy versus Diploidy in the Reproduction of Cell Type

WALTER TULECKE

Introduction 217
Characteristics of Haploids 218
Induction of Haploidy 221

Radiation Effects 223
Quantitation and Biochemistry 223
Growth and Differentiation 225
Haploid Tissue Cultures of Higher Plants 229
Conclusion 236
References 238

Cell and Tissue Interactions in the Reproduction of Cell Type

IRWIN R. KONIGSBERG AND STEPHEN D. HAUSCHKA

Introduction 243
Regulation of Stem Cell Differentiation 245
The Cellular Environment and Stem Cell Differentiation 246
Experimental Approaches to Embryonic Induction 248
Cytodifferentiation in Cell Culture 249
Population Density and Cytodifferentiation 252
Conditioned Medium and the Development of Muscle Clones 253
The Conditioning Process 254
Biological Parameters of Conditioning 256
The Critical Period 260
Conditioning the Petri Plate Surface 262
Collagen Synthesis during Conditioning 268
A Hypothetical Role of Conditioned Medium 273
Macromolecules and Cell Culture 276
Collagen and Differentiation *in Vivo* 279
Summary 284
References 286

Aging As a Consequence of Growth Cessation

ROBERT R. KOHN

Introduction 291
Scope of Aging 292

Growth and Aging 294
Animal Aging 295
Aging of Cells within Animals 313
Summary 319
References 321

Author Index 325

Subject Index 337

Reproduction: Molecular, Subcellular, and Cellular

Reproduction: Molecular, Subcellular, and Cellular

HERBERT STERN*

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Introduction

The purpose of this symposium is partly to clarify and define specific aspects of reproduction at different levels of biological organization, and partly, in common with other symposia held by the Society, to stimulate interaction between various intellectual approaches to a broad phenomenon of common interest. Whether or not a cell reproduces true to parental type, or whether a cell reproduces at all, are issues of fundamental interest in developmental biology. Reproduction of a cell is impossible without reproduction of molecules, but the kinds of molecules reproduced and the conditions effecting reproduction are dependent upon the intracellular pattern of molecular organization and the influence of the environment on the metabolic poise of the cell. The production of a chlorophyll molecule, for example, may be fully stated in terms of the enzymes in its biosynthetic chain. The reproduction of a chlorophyll molecule is, however, quite a different matter. A necessary condition for reproduction is the *transmission* of proplastids or plastids from parent cell to offspring. Even if such transmission occurs, the production of chlorophyll remains dependent upon the location of the cell within an appropriate region of the plant. Thus, excluding environmental and mutational factors, a clarification of the phenomenon of chlorophyll reproduction requires the elaboration of certain rules governing the behavior of subcellular organelles with respect to transmission, as well as rules governing the development of organelles with respect to cell differentiation. A counterpart of this example may be found in the reproduction of many other cell components. The involvement of a cytoplasmic particle is incidental to the general question of reproduction. Applicable to all situations, however, is the fact that just as the reproduction of cells does not assure the reproduction of all molecules, so the reproduction of molecules does not assure the reproduction of cells. We cannot avoid the

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challenge of defining rules for the formation of molecules within the biosynthetic framework of cells, for the assembly of molecules within the architectural framework of cells, and for the reproduction of molecules or their complexes within the genetic framework of cells as governed by the interaction between cells and their environment.

We may justifiably make the dogmatic assertion that a complete understanding of cell reproduction requires a complete understanding of the molecular events underlying such reproduction. We cannot, however, extrapolate from molecular behavior to cell behavior without guidance from the cells themselves. A molecular mechanism, to the extent that it is perfectly understood, imposes clear restrictions and suggests fruitful possibilities with respect to the elaboration of rules concerning reproduction at more complex levels of organization. But unless we make the extremely doubtful assumption that the present organization of living systems is a necessary and inevitable consequence of molecular properties, we cannot hope to achieve a knowledge of reproduction by a unidirectional acquisition of information from molecule to man. Even if we allowed for this theoretical possibility, we would have to atribute to biologists a flawless prescience never witnessed previously in the history of science. Were it not for the discovery of mitosis, meiosis, and Mendelian inheritance, the Watson-Crick helix would not have come into existence; and, if it were somehow envisaged, it would have remained a conversation piece. If the molecular biologist must be defined as one who chooses problems which can be solved, then the biologist must be defined as one who chooses problems which need to be solved. These appositions and oppositions are, however, trivial. The point of view that clarity of formulation is essential in approaching the phenomenon of reproduction at all levels of organization represents the spirit in which this symposium was fashioned.

We are generally concerned with two aspects of reproduction, the mechanisms themselves and the regulation of these mechanisms. Although a distinction between these two aspects is often difficult to make, we may nevertheless attempt to outline the structure of the symposium with this distinction in mind. We should be able to define reproductive mechanisms at the molecular, subcellular, and cellular levels with some degree of rigor. Similarly, we should be able to define molecular, subcellular, and cellular regulatory mechanisms.

Molecular Mechanisms of Reproduction

Our present concepts of molecular reproduction are based entirely on two fundamental principles, enzyme catalysis and complementary nucleo-

tide pairing. The first is an oldtimer in biology; the second is a newcomer. The enthusiasm which greeted the discovery and studies of enzymes in the late nineteenth century is now focused on investigations of the function of nucleic acids. Whether molecular biology begins with base pairing, or dates back to the studies of Claude Bernard, is a matter of pride and prejudice with no relevance to the symposium. Our interest lies in the fact that enzymes select from the thermodynamically possible world and construct the real world of living matter. Bond formation without enzyme mediation is an event which can at best be of only marginal interest in biological reproduction. All molecules are synthesized by virtue of specific enzymes. But the very specificity of an enzyme, the relatively small intramolecular distances which its active site can recognize, precludes its operation in specifying the order of extended intramolecular sequences. To this particular aspect of ordering nucleic acids furnish an essential contribution.

Three categories of molecular synthesis have been considered in this symposium—autosynthesis, heterosynthesis, and antibody synthesis. Whether the categories are variants in the operation of enzyme catalysis and nucleic acid pairing, or whether other mechanisms are yet to be disclosed, remains to be seen. At present, we feel confident that the two basic mechanisms described are sufficient to account for all forms of molecular biosynthesis. In autosynthesis, an enzyme transcribes a complementary base sequence out of subunits identical with those of the template. This type of synthesis is considered universal in DNA and operative in RNA only in the case of RNA viruses. In heterosynthesis, an enzyme also transcribes a nucleic acid template, but in the case of protein synthesis, the transcribed product must be translated into a sequence of amino acids by a different set of processes. Antibody synthesis may be *formally* described as the formation of proteins complementary to antigen molecules. The discussion of this topic by Nisonoff should make it amply clear, however, that formal identities between nucleic acid complementarity and antigen-antibody complementarity have no counterpart in molecular terms. Present evidence virtually rules out the possibility that an antibody molecule may be synthesized by a transcription of antigen. Doubt even exists as to whether the total sequence of molecular events, from antigen challenge to antibody production, is encompassed by a single cell. It would appear that rules must first be formulated at the cellular level before molecular mechanisms can be properly resolved.

The two basic molecular mechanisms, if rigorously examined, reveal certain limitations and some possibilities with respect to molecular reproduction. On the assumption that the mechanisms are properly under-

stood and that no other mechanisms exist, direct copies of molecules are ruled out. Since sequences are transcribed by base pairing, the primary product of transcription must always be complementary to the template. The molecular distinction between autosynthesis and heterosynthesis is blurred, inasmuch as the primary determinant of the subunits used in transcription (whether ribo- or deoxyribonucleotide) is the enzyme. Nothing in our present concept of nucleic acid reproduction excludes the possibility of RNA being transcribed into DNA. Our failure thus far to discover instances in which DNA is replicated from an RNA template, or in which native cellular RNA is transcribed from RNA templates, cannot be sanctioned by molecular rules of reproduction. Moreover, since the agent of transcription is the enzyme, we must also allow for the possibility that direct rather than complementary copying may occur if the appropriate enzyme is found. Either the rules must be tightened, or our knowledge of cell behavior must be broadened. We may, on the other hand, dogmatically assert that the amino acid sequences within a polypeptide chain can only be derived from nucleotide sequences and that the reciprocal derivation is impossible. We are thus compelled to account for the reproduction of protein molecules in terms of nucleic acid templates.

Subcellular Mechanisms of Reproduction

At the subcellular level, the existence of a supply of molecules is taken for granted and the question to which we must address ourselves is how the various structures in a cell are reproduced from the molecular pool. The presence of DNA templates in the cytoplasm mainly influences the phenomenon of intracellular regulation. The geography of transcribing systems is largely, though not entirely, incidental to the mechanisms involved in the generation of subcellular structures. Our knowledge of these mechanisms has none of the crispness characteristic of our present understanding of molecular events. Currently, we envisage three possible mechanisms in the formation of structures from molecular pools: self-assembly, accretion by intussusception or end addition, and assembly on preformed templates. The first of these has evoked considerable conceptual interest because of the implication that individual macromolecules contain sufficient information to arrange themselves in recognized biological patterns. Bacterial flagella and collagen fibers are among the structures thus studied for which the evidence pertaining to self-assembly is impressive. The biological consequences of such a process are clear. If the information contained in a coded amino acid sequence is sufficient to determine the tertiary structure of a macromolecule, and also to assemble

that molecule along with others similarly endowed into a discrete subcellular structure, then subcellular reproduction is an obligatory consequence of molecular reproduction. Even if allowance is made for the possibility that enzymes might catalyze the self-assembly process, a passive role would still have to be assigned to extragenic components in the generation of subcellular forms.

Whether the concept of self-assembly can be extended to all formed elements in a cell remains to be seen, but the prospect for such extension is, at least in some instances, not bright. "Membranes" have been generated by modifying the old technique of mixing lecithin with water to create myelin forms. Modification consists of the addition of protein to the initial mixture and observation of the product under an electron microscope. The artificial model is undoubtedly similar in appearance to a natural membrane. Unlike the self-assembled structures discussed above, however, membranes are transmitted as such from one cell generation to another; a membraneless cell is unknown. Where careful observations have been made of the formation of specific membranes, as in the chloroplast, evidence points to the growth of new membranes from preexisting ones. The functional and structural heterogeneity of membranes (e.g., inner membranes of mitochondria, lamellae of chloroplasts, limiting membranes of cytoplasm) apparently makes self-assembly from a pool of individual molecules a rather hazardous morphogenetic operation. The issue is whether the essential genetic complement of a cell is reducible to sequence coding, or whether it must also contain other preformed elements that serve as frameworks, however small and undistinguished, for the reproduction of recognized structures. Cortical inheritance in protozoa furnishes the best-studied example of such a requirement. Other examples of cytoplasmic inheritance (discussed by Srb) may be attributed either to a requirement for the transmission of preformed cytoplasmic structure or as elucidated by Schiff, to the presence of cytoplasmic DNA.

The reproduction of chromosomes is clearly not a process of self-assembly. The problem of chromosome reproduction is unique inasmuch as it is preceded by DNA replication, and the formation of the longitudinally differentiated structure as an apposition of materials to a preexisting DNA skeleton could be rationalized with some theoretical and empirical justification. Nevertheless, difficulties remain both in defining the actual molecular organization of the chromosome and in explaining how its characteristic cytochemical morphology is maintained. Swanson has pointed out that a single double helix of DNA is still a favorite model for the primary skeleton of the chromosome, although this repre-

sentation is not universally embraced. Modifications of the model have to be made in order to account for the many starting points of replication within a single chromosome. The acquisition of genetic data, inadequate as the data may be, would be helped rather than hindered by postulating the existence of specialized linkers between gene segments. Whatever the precise nature of the DNA skeleton, thought must be given to the mechanisms which confer upon that skeleton its full structure. Are histones deposited randomly, or are there localized sites in the DNA filament which interact with specific histones? If the latter is true, we must envisage a mechanism by which segments of a DNA chain recognize specific histones. Is the RNA in a chromosome a collection of unspent messages destined for the cytoplasm, or does it have some special function in making chromosomal proteins which are properly apposed to the DNA filament because they are locally synthesized? Curious though it may seem, the major problems in understanding chromosome reproduction do not center around the replication of DNA. Our preoccupation with nucleic acids and their potentialities in specifying macromolecules overshadows these other problems but does not diminish them. The fact remains that the reproduction of a chromosome is not implied in the replication of DNA. Only future studies will tell whether a total implication is even possible.

Mechanisms of Cellular Reproduction

In defining mechanisms of reproduction at the cellular level we must take for granted the operation of reproductive mechanisms at the molecular and subcellular levels. If this is done, we might wonder whether anything can be said about cell reproduction. Little in our experience leads to the view that an analogy can be drawn between the formation of subcellular organelles from molecules and the formation of cells from subcellular organelles. Indeed, the view that cells are aggregates of functionally integrated but structurally discrete systems is almost universal. As a consequence of this view, we are inclined to define the problems of cell reproduction entirely in regulatory terms. Nevertheless, however correct this view, one aspect of cell reproduction—the partitioning of subcellular components—cannot be considered except at the level of cell organization. If nothing else were said about this complex problem, we would have to concede that a mechanism hitherto unmentioned—polarity—is a necessary component of the reproductive process. This does not imply that polarity is characteristically a supramolecular property which emerges only at the cellular level of organization. Indeed, a polarity

certainly exists in the structure of every nucleic acid and phospholipid molecule; polarity almost certainly exists in the myosin molecule and most probably is to be found in many types of proteins. The existence of polarized molecules is a necessary but hardly sufficient condition to account for the polarized movements of components which occur during cell reproduction.

Partitioning of a cell into daughter cells does not involve a polarized migration of all components. Obviously, some components do not move. Moreover, it is questionable whether subcellular populations occurring in relatively large numbers (chloroplasts, mitochondria) require equipartitioning to achieve equivalent numbers in the matured daughter cells. In *Euglena,* as pointed out by Schiff in the course of discussion, the imbalance created by a partial loss of proplastids or chloroplasts is eventually relieved by their reproduction at a compensatory rate. Nevertheless, although many components do not require polarized movement, it is apparent that chromosomes do. Indeed, one may extend this requirement even to prokaryotic organisms. Replication of DNA does not in itself account for equipartitioning, and without such partitioning genetic continuity could not operate. We may not have sufficient experimental and conceptual tools to cope with the problem of polarity, but one cannot avoid the impression that this phenomenon is commonly regarded as an eccentricity rather than as a profundity of natural organization. We must also recall that the role of polarity in reproduction is not a singular one. Meiosis and mitosis lead to completely different modes of cell reproduction, and one of the major differences between these two similar processes depends upon the packaging of polar movements. The separation of chromosome pairs in meiosis is probably the most outstanding example of differentiation effected by unequal partitioning of cell components. The once strongly held view that differentiation may arise from an unequal partitioning of components among daughter cells has lost its impact. Nevertheless, the fact remains that in at least some instances (stomatal formation, microspore maturation) unequal division of a cell leads to daughter cells with differentiated functions.

Regulation of Reproductive Mechanisms

Regulation is so diffuse a subject that changing fashions in its investigation are to be expected, the more so, since one good scheme can explain a lot without proving much. We might have focused exclusively on the regulation of gene action in relation to reproduction. The topic would

be timely, and carry the full prestige of profundity, but in doing so we would have chosen the less complex issue and avoided the more difficult challenge. On the other hand, it is doubtful whether we are in an effective position to take on the challenge. An attempt has been made in this symposium to classify regulatory mechanisms in terms of organizational level. Such sorting out should help to clarify our thinking and, hopefully, to emphasize the inherent limitations of any single control mechanism. In an integrated system such as a cell or organism, the profound mechanism may turn out to be the most invariant one, and the apparently trivial mechanism the one most sensitive to environmental fluctuations and hence the most important in the translation of subtle influences into profound reactions.

Molecular Regulation

At the molecular level, the regulation of reproduction can be defined in terms of the two much discussed mechanisms, complementary base pairing and enzyme catalysis. The first of these has lent itself to a variety of schemes all based upon the principle that linear nucleic acid templates must be transcribed directionally. Evidence that transcription of one nucleic acid molecule into another and translation of the latter into protein proceeds directionally, rather than by a simultaneous or random apposition of complement, is strong enough to be adopted, tentatively at least, as an axiom of the process. Direct evidence has been obtained from studies of protein synthesis and from tracing the reproduction of genetic markers in mocrobial systems. The question mentioned by Atwood—whether transcription and translation occur in the same or in opposite directions—is important in detailing the mechanism, but does not affect the validity of linear reading as a regulatory device. The general evidence naturally invites the conclusion that the prime target of any regulatory mechanism is the starting point of transcription. Blockage or opening of the starting point encompasses all the schemes involving repressors, derepressors, operators, and regulators. Such schemes lend themselves to an infinitude of designs, and proof rather than explanation is the principal missing ingredient. The main problem, however, is not to prove the formal validity of a scheme too logical to be abandoned. It is on the more restricted issue of molecular recognition that the scheme requires investigation. For in a very real sense, the nucleic acid filament, although it is replete with information about sequence, appears to be rather static in its behavior. Unless we misunderstand its operation, its regulatory role must be confined to a choice between being read or not being read. Even so,

the choice must be effected by some substance which is not a part of the nucleic acid tape. Such a substance could be the direct product of a gene, but directly or indirectly it must certainly be the product of events in the cytoplasm. The case of the substrate inducer in microorganisms is now classical, but it takes little imagination to perceive that the possibilities of similar relationships are virtually unlimited in a multicellular system.

If we postulate that promotion of transcription for a defined portion of the nucleic acid filament is a function of the attachment between the transcribing enzyme and the starting region of the filament, a variety of conditions may be considered necessary for molecular biosynthesis. If the enzyme is lacking, no transcription will ensue. If the enzyme is present but the starting site is complexed with some other substance (e.g. a polynucleotide or protein), transcription will also be blocked. If another substance with a higher affinity for the blocking agent than the polynucleotide segment is produced, the block will be removed. This is essentially the repressor theory of regulation. Modifications of this scheme are clearly possible. The evidence that chemical alteration may occur in the intact chain (e.g. methylation) as a result of the action of specific enzymes, opens a new arena for speculation. Given the inherent linear orderliness of the nucleic acid molecule and the capacity of enzymes to recognize localized segments, we can visualize a variety of enzymes, activated under a variety of conditions, effecting specific changes in certain nucleotides which enhance the capacity of the chain to recognize and interact with specific substances in the environment. This possibility has been considered by Atwood and, in a somewhat different form, by Dulbecco who postulated the operation of a nuclease in effecting specific breaks in the chain.

Whatever our preferences for regulatory schemes, it is apparent that linearity has consequences for the cell which extend far beyond the coding of amino acid sequences. Granick has indicated in his comments that the amount of DNA per cell in higher organisms cannot be rationalized on the basis that such organisms effect more biosyntheses than do bacteria. The fact that regulation of molecular reproduction is far more extensive in higher organisms than in bacteria, however, may offer a possible explanation. But if we postulate more DNA lengths for regulatory purposes, we must also postulate more proteins and/or other substances which are the active components of any regulatory system.

All our information points overwhelmingly to protein molecules as the most sensitive receptors of intracellular or extracellular environmental stimuli. In many cases the protein molecule may be associated

with a chromophore or other prothetic group, but such an association may be regarded as an extension of its powers. If regulation were no more than synthesis or lack of synthesis of macromolecules, we could assign to proteins a purely supporting role. But the evidence is almost entirely against so narrow a definition. From the start, development is a process of eliciting the expression of genetic potential. Much, if not all, of that potential is linearly taped, but the tape states what can be done whereas the environment of that tape states what will be done. At the molecular level we have yet to learn how environment and tape interact.

Subcellular Regulation

If regulation of reproduction at the molecular level encompasses considerations well beyond the immediacies of the molecules involved, difficulties in drawing any clean line between regulation at the subcellular and cellular levels should not come as a surprise. The distinctions we make must be arbitrary and can be justified only by the extent to which they have some operational meaning. With this in mind we may say that the major impact of regulatory mechanisms at the subcellular level must be in the coordination of relative population numbers. In a clone of nondifferentiating cells this means equivalent rates of reproduction among all cellular constituents from one generation to the next; in the case of a differentiating clone this often means alterations in otherwise equal rates of reproduction. We must distinguish, however, between subcellular components having their own genetic continuity and those which do not.

For subcellular components lacking a genetic continuity of their own, regulation can be considered only in the same terms applied to molecular components. On the other hand, for subcellular components with their own genetic continuity we may at once define two requirements: Their transmission to daughter cells must be assured and their net rates of reproduction must equal that of the nucleus. In cases where unequal segregation occurs, an additional mechanism must be present to restore the original numbers. If these mechanisms do not operate then, the progeny undergo differentiation. The two mechanisms involve both cytoplasmic inheritance and cell differentiation. The various examples of cytoplasmic mutation discussed by Schiff and by Srb invite us to consider not only molecular mechanisms but also subcellular ones. The chromosomal system is so organized that mutated genes are replicated and transmitted like their counterparts. So far as we know, this relationship does not hold for cytoplasmic systems. A mutation within a chloroplast,

for example, must be evaluated from the standpoint of two parameters: (1) the effect on development and on functions within the cell and (2) the effect on reproductive rate relative to other chloroplasts and to the cell as a whole. Only mutations which have no depressing effect on reproductive rate would survive, and those which elevated the reproductive rate would eventually displace the original population. We know too little about the relations between physiological function and reproductive rates to draw any general conclusions, but we may suspect an interaction between the two properties. Such an interaction would serve to stabilize the characteristics of cytoplasmic components with genetic continuity. The implications of this interaction are perhaps forgotten in pondering the significance of cytoplasmic DNA. If it can be assumed that such DNA does serve as a coding template and in this respect is identical with chromosomal DNA, an important distinction is to be found at the subcellular rather than at the molecular level. A single mutation within a chromosome is perpetuated for as long a period as the cell line itself; a single mutation within a cytoplasmic particle is perpetuated only if its reproductive rate remains unaffected by the mutation. From the standpoint of evolution, chromosome structure is the more effective vehicle.

The observations of Tulecke on the respective growth patterns of male and female haploid tissues raise an important question which invites exploration. The fact that female tissues contain not only chlorophyllous cells but also various other differentiated elements lacking in male tissues, suggests that their respective cytoplasms have different morphogenetic potentials. On first inspection, we cannot attribute these differences to the genome and are therefore compelled to consider the cytoplasm as a seat of heritable factors relevant to the differentiating process. If this is true, then totipotency cannot be considered entirely in terms of the genome. The hazards in simple interpretations of cytoplasmic inheritance have been stressed by Srb; this caution should be respected but not overemphasized in evaluating the behavior of haploid plant tissues.

The regulation of chromosome reproduction still remains a problem of major interest. The problem is somewhat simplified by our assumption that DNA provides a structural template to which other constituents must be apposed. The simplification permits us to probe some aspects of chromosome reproduction even though we are uncertain about the nature of the structure to be reproduced. Evidence that the mere presence of essential enzymes and substrates is insufficient to initiate chromosome reproduction is strong. We are therefore led to consideration of the remaining molecular mechanism—the exposure of starting points on the

template. Excluding, however, this particular mechanism which relates specifically to DNA, we are still uncertain, as Swanson has pointed out, about the regulation of other chromosomal components. We frequently make the tacit assumption that the macromolecules in a chromosome are reproduced in the same way as other cellular macromolecules, but this conclusion is something less than a fact, and we have yet to discriminate between nuclear metabolism which serves the cytoplasm and nuclear metabolism which serves the chromosome. We know from cytological data that DNA replication is not an assurance of chromosome reproduction. If it were, polyteny would be unknown. We must also elucidate the regulation of centromere reproduction, for in its control lies part of the answer to meiotic and mitotic reproduction. Moreover, to these difficult considerations another one which emerges from studies of viral infection must be added. However autonomous a replicating system appears to be, the introduction of another replicating system into the cell may lead to interactions which affect the rate of chromosome reproduction. If such effects occur between virus and genome, they may also occur between genome and normal cytoplasmic constituents.

The clarity which can be given even to partially proved schemes of molecular regulation stems from the fact that multiple interacting systems may be conveniently ignored. That multifactorial considerations enter, however, is only part of the problem. The other part relates to the obvious fact that we do not know enough about the mechanisms by which the subcellular structures are reproduced. Since our knowledge of the pure mechanisms of reproduction is uncertain, we can hardly expect to clarify the regulation of those mechanisms. We are well aware that in a developing organism we must deal not only with factors that assure reproduction, but also with factors that assure selective reproduction. At this moment, these factors require a much better understanding of how structures are assembled, rather than how frequently or under what circumstances message RNA is produced.

Cellular Regulation

If we turn from the subcellular to the cellular level of reproduction we are at once faced with a fact, too often ignored, that each cell has a history, and that whatever the modes and mechanisms of subcellular reproduction, cellular events occur in a characteristic pattern along the axis of time. Presumably, the information for temporal organization, just like that for spatial organization, is encoded in the hereditary apparatus. The challenge is not to prove the assumption, but to clarify the unfolding

of this temporal pattern. Studies of viral reproduction suggest that the reference axis for temporal behavior is in the axis of the DNA filament. Directional reading of a filament thus assures a sequential expression of events. Yet even if we accept as a certainty the occurrence of such a translation of temporal pattern, it is clear that the mechanism cannot be extended to the behavior of a whole cell, if for no other reason than the fact that cells may have large numbers of chromosomes, and in order to maintain total directional reading, gap messages would be required to trigger chromosomes sequentially. A sequence of chromosome readings is nevertheless open to question, since numerous translocations and inversions are known which do not have any marked effect on the developmental cycle of a cell. The possibilities for regulatory schemes in a system consisting of a linear template with a controllable starting point are unlimited, however, and a satisfactory temporal scheme could easily be designed out of DNA filament readings no matter how many discrete filaments we postulate for each cell.

Despite the fact that we can grossly ascribe to every cell an inherited temporal pattern of development, the pattern must be much more rigorously defined before it is subjected to meaningful study. The only operational value in ascribing the characteristic of temporal patterning to a cell, lies in being able to assert that events a,b,c,d, etc., must occur in sequence. Moreover, the order of such events must remain unaffected by environmental factors. We expect the environment to affect the rate of transformation, to interrupt it, or to initiate it, but not to change the order of occurrence. If we now ask ourselves which aspects of cell history have thus been adequately analyzed, the answer must be somewhat equivocal. The common target has been the "cell cycle" occurring in the sequence G_1SG_2M. There is no question that this cycle is common. Yet we know from some studies that the S period may occur in the telophase of division, thus preceding the G_1. We also know that the common characteristics of the G_1 phase—protein and nucleic acid synthesis to the point of doubling the initial cell size of the daughter cell—may hardly occur in all even though a G_1 interval exists. In male gametogenesis in plants the microspores resulting from meiosis show little synthesis of nucleic acid or protein during an extended G_1 period, but form ribosomal RNA and protein only close to the time of DNA replication. And we already know from classical cytological studies that DNA synthesis need not be followed by mitosis.

Thus, if we examine the most thoroughly studied type of temporal pattern, the findings in support of a predetermined history are meager.

The only assertion we can make is that DNA synthesis must precede mitosis. Any of the other developments which apparently occur in a fixed sequence could be rationalized by assuming that one of the consequences of mitosis is an imbalance in the ratio of chromosomal to non-chromosomal components, and that such an imbalance is removed by the complex operation of feedback systems. The denial of a predetermined history to a system which repeatedly reproduces the same history may appear absurd, yet the absurdity may lie in our attempt to reveal a 1:1 correspondence between discrete physiological events and a linear tape. Perhaps the only linearly taped history is that of an operon as defined by Jacob and Monod.

We may relieve ourselves of the task of resolving the characteristics of a taped history, but we cannot relieve ourselves of the fact that each cell and each organism does have a finite history. If so, we must ask ourselves how such a history, whatever its motive source, is related to the problem of regulating cell reproduction. Clearly, no single cell goes through life by reading its chromosome filaments progressively. In a proliferating clone, a cell reads a fixed length of linear scripture and its daughters repeat the exercise. In a differentiated cell, a novel piece of text is read, but the end of the text does not coincide with the end of the cell. For a greater or lesser interval after the first reading, the cell eschews any new theology and retains the familiar text for re-reading. Furthermore, cells of all types have a common genetic text to provide for common requirements—energy transformation, carbon chain building, active transport, and mechanical work. How frequently a cell must consult its tape is a question which currently takes the form of a search for stable message. How readily a cell may be induced to transcribe a segment of its tape is a question which is now often framed in terms of the elicitation of RNA by hormones. Such studies may have overlooked the fact that the genetic tapes of cells at different developmental stages and in different developmental forms need not have identical responses to the same substance. The transcription of some segments may be open to stimulation; the transcription of others may not be. We do not know how the mechanisms of opening and closing operate, but we may hazard a prediction that such mechanisms involve the history of the cell. That history is embedded in an interaction between cell and environment, and the consequence of that history is to provide for a variety of cumulative changes within the cell. Such changes would be expected to filter environmental stimuli and hence affect the cellular response pattern.

The discussion of Konigsberg points to the superfluity of certain profundities and reasserts the historic experience that the apparently trivial may be pregnant with profound implications. His principal target has been the differential reproduction of myoblasts, and he has sought to clarify the rules governing such reproduction. In seeking the pertinent rather than the profound, he has discovered, in common with others, that extracellular collagen may be a causative factor in differentiation. The line of communication between collagen and message may be long or short, but studies of this type point to the epistemological value of the clear fact as opposed to the hazy doctrine.

In a similar vein, one must take into account the provocative discussion of Kohn on the aging process. Some stress mutation of the template; others stress deterioration of the individual cell. Kohn stresses neither; his emphasis is on the organism which represents an integrated community of cells. Any process which interferes with the integrity of intercommunication is a potential factor in aging. Such interference may of course occur by virtue of the deterioration of certain cell types, brain cells, for example. Unequivocal evidence is not available, but Kohn does present some convincing evidence that the functional capacities of several cell types do not deteriorate with age and that the villain is a progressive alteration in collagenous material. A full reading of this text would lead to the conclusion that immortal organisms are stayed from immortality because their cells are mortal, and that immortal cells are stayed from immortality because their host organisms are mortal. The conclusion may offer little psychological comfort but it does afford intellectual stimulation.

We are still very much in need of some bold strokes which will sketch the capacities and limitations of the reproductive abilities of cells. We must be able to distinguish between flexibility of differentiated cells to adapt to varying environmental pressures and their flexibility to resume a novel life history. Ultimately, all the footnotes to the major phases of a cell's history will contain molecular explanations, but the process of learning is such that the history must be known before the footnotes are written. This symposium should tell us not only how well prepared we are for the footnotes, but also how well prepared the footnote writers are themselves.

These comments do little to reflect the alacrity of discussion at the symposium. The genesis of the alacrity must be attributed to a blend of circumstances: the personalities of speakers and chairmen, some per-

versely colorful, others poetically serious; the avid responses of the audience; and the ebullient hospitality of our host, Carleton College, which cemented social *gemutlichkeit* to intellectual venture. To all encompassed by these circumstances the Society expresses its deepest appreciation.

REPRODUCTION AT THE MOLECULAR LEVEL

Transcription and Translation of Genes

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This review concerns the formation of cell constituents, RNA and protein, that are colinear with DNA. The segment of DNA with which such a constituent is colinear will be called the gene for that constituent. Transcription denotes the copying of a DNA nucleotide sequence into RNA; and translation denotes the synthesis of polypeptides in which the amino acid sequence is specified by an RNA nucleotide sequence in accordance with the genetic code. These processes, which constitute gene action, are relevant to the problem of differentiation among cells of identical genotype, the old familiar problem that remains central in developmental biology.

ur-Genes and the Gene Action System

Gene action is accomplished by means of a special set of components, the gene action system, essential to organisms without any known exception. The system comprises three categories of RNA and a number of different proteins. At least 24 proteins are required, but there is no adequate reason at present for proposing that there are more than 89. We may refer to the DNA segments that specify these components as *ur*-genes; their ubiquity and their necessary functional relation to all genes suggests that they had already evolved into definitive form in a common ancestor of the known biota.

A minimal biological system could, in principle, be based solely on *ur*-genes. It would be devoid of intermediary metabolism and therefore would require that the 20 amino acids, 4 ribonucleoside triphosphates, and 4 deoxyribonucleoside triphosphates be present in the environment. Its sole activity would be the assembly of these substances into genes and into the components of the gene action system. A description of this hypothetical minimal system and an enumeration of its components will

provide the preliminary frame of reference for a discussion of the processes of transcription and translation.

A fundamental aspect of the organization of the gene action system is that, except among RNA viruses, DNA is the only part of the system that specifies itself. It follows as a logical necessity that DNA also specifies all the other polymers; DNA does not, however, synthesize itself. An enzyme or enzyme complex is needed for the duplication of DNA, and in order to synthesize an enzyme specified by the DNA, transcription and translation must take place. Transcription requires a transcribing enzyme, the RNA polymerase, which synthesizes the RNA messages (mRNA) to be translated, and also synthesizes the RNA components of the translating system. The latter components are the transfer RNA (tRNA), or genetic dictionary, and the ribosomal RNA (rRNA). A reasonable estimate of the number of components in the complete system can be made from its known properties.

The Ribosomal Components

A ribosome can be analogized to a mechanical tape-reading device that moves from one end of the genetic message to the other. Typically, it contains two molecules of RNA distinguishable by their sedimentation constants, e.g., in *E. coli* a 16 S and a 23 S, and in a number of metazoans an 18 S and a 28 S fragment. The rRNA alone is not sufficient to make a functional ribosome; about 35% protein is normally present. About 20 different protein fractions can be obtained from ribosomes. The problem remains of distinguishing those of adventitious occurrence from those that are indispensable constituents. Streptomycin resistance, a property of the ribosomes (Davies, 1964; Flaks *et al.*, 1962), is probably a modification in ribosomal protein since its genetic locus is different from the locus for rRNA. The number of ribosomal proteins thus lies between the limits of 1 and ca. 20.

The Transfer RNA

The tRNA with a molecular weight of about 25,000 and a sedimentation constant of 4 S has two specific recognition sites: the anticodon which matches complementary trinucleotide codons of the message, and, a site recognized by a transfer enzyme that is in turn specific for the amino acid signified by the codon. Thus, the minimum number of kinds of tRNA is 20; that is, 1 for each amino acid. The maximum number is 64, the number of codons that can be formed from 4 bases. The actual number is determined by the number of synonyms for amino acids, in other

words, by the degree of degeneracy of the code. If, for example, a given amino acid has three synonyms in the codon catalogue, it must have three kinds of tRNA, each bearing the anticodon to a different one of the synonymous codons. The different experimental approaches to the construction of a complete catalogue of codon assignments, e.g., mutational amino acid replacement (Yanofsky, 1963; Wittmann and Wittmann-Liebold, 1963) and trinucleotide-directed formation of charged tRNA-ribosome complexes (Nirenberg and Leder, 1964), concur in suggesting that the number of codons that signify intercalated amino acids, and hence the corresponding number of kinds of tRNA, is near the upper limit.

The Transfer Enzymes

The number of transfer enzymes similarly lies between the limits of 20 and 64. In contrast to the number of tRNA's, however, the number of transfer enzymes is not necessarily determined by the number of synonyms

TABLE I
COMPONENTS SPECIFIED BY THE *ur*-GENES

Component	Minimum number	Maximum number	Most likely number
Duplicase	1	2?	2
Transcriptase	1	1?	1
Peptide polymerase	1	2?	2
Transfer enzyme	20	64	20
Ribosomal protein	1	ca. 20	2
Total proteins	24	89?	27
rRNA	2	2?	2
tRNA	20	64	64
Total number of *ur*-genes	46	155?	93

for the amino acids. It is not yet known whether tRNA molecules with synonymous anticodons have the same enzyme recognition site. If they do, then the number of transfer enzymes could be 20 even when the number of different tRNA's is 64. Nucleic acids (especially tRNA) undergo specific modifications such as enzymatic methylation of certain bases. On the assumption that such modifications are not essential to the functioning of the gene action system, the foregoing enumeration of components may be summarized in Table I.

It is noteworthy that these components are formed by the same processes for which they themselves appear to be essential. Our ignorance of the many intermediate stages in their evolution remains complete.

The Transcription Process

The transcribing enzyme, or RNA polymerase (Spiegelman, 1958; Stevens, 1961; Geiduschek *et al.,* 1961; Chamberlin and Berg, 1962; Furth *et al.,* 1962), works with ribonucleoside 5′-triphosphates and a template which normally is double-stranded DNA. The polymerization proceeds, as in the duplication of DNA, with the release of pyrophosphate and the formation of the phosphodiester linkage between the 3′- and 5′-positions of adjacent nucleotides. The double helical DNA template shows no change as a result of its use in transcription, and the precise nature of the pairing between RNA and DNA at the growth point of the RNA chain is problematical. It is generally assumed that base pairing is required at this point to insure the production of an RNA copy that is complementary to one of the DNA chains. If so, some form of transitory local separation of the DNA strands would be required at the point of transcription. Transcribing enzyme will also work with a single-stranded DNA template (Chamberlin and Berg, 1963; R. C. Warner *et al.,* 1963), but the result in this abnormal situation is the formation of a complementary RNA strand which does not separate from the DNA, but remains in a very stable hybrid double helical configuration. Under the abnormal conditions of some *in vitro* systems, the transcribing enzyme will work on an RNA template (Nakamoto and Weiss, 1962; Krakow and Ochoa, 1963) to produce a complementary strand which remains in an RNA double helix. This property of the transcribing enzyme may have caused some confusion in the identification of special RNA-replicating enzymes specific for RNA viruses.

The normal transcription process exhibits two very important features that tend to be obscured in the *in vitro* experiments. The first of these is strand selection (Hayashi *et al.,* 1963b; Tocchini-Valentini *et al.,* 1963). Complementary RNA strands are not normally found in cells; hence, we may speculate that only one of the complementary strands is produced, or that both are produced and one is selectively degraded. The experimental evidence indicates that the selectivity is at the level of transcription itself, rather than in a subsequent degradation. *In vitro* systems ordinarily produce complementary RNA, but if special precautions are taken to obtain template DNA that has not been subjected to hydrodynamic shearing, the product RNA *in vitro* is asymmetrical just as it is in the cell. Strand selectivity in such systems is lost in proportion to the degree of mechanical breakage of the DNA, suggesting that the broken ends of the DNA provide points of initiation from which the

transcription of the "wrong" DNA chain can proceed. The nature of the distinguishing characteristic at the point of strand selection is one of the outstanding unsolved problems; the solution may be related to that of the second ubiquitous and important feature, chain delineation.

In normal transcription an RNA molecule is produced that begins and ends at predetermined and reproducible points, although the corresponding template DNA is a segment contiguous with a much larger DNA chain. The molecular basis for chain delineation, like that of strand selection, is unknown, but it must ultimately reside in local sequence characteristics of the DNA. One hypothesis is that specific sequences for beginning and ending transcription are recognized by transcribing enzyme. This encounters difficulty in explaining the readiness of transcribing enzyme to accept the erroneous beginning points in fragmented DNA. Alternatively, the enzyme does not recognize sequence, but transcription boundaries may be delineated by local base separation, single-strand breaks, or the presence of specific non-DNA entities. If such a hypothesis is correct, then the agency that produces such a local effect must recognize the local sequence, since sequence is, ab initio, the only thing that distinguishes one part of a DNA chain from another.

The Translatable Message

The mRNA, traditionally called "messenger," (Brenner, 1961; Brenner *et al.,* 1961) comprises the RNA chains which are normally translated into protein. Unlike the foregoing components, it is characterized by a base composition similar to the DNA, and usually by a very rapid turnover (Gros *et al.,* 1961; Hayashi and Spiegelman, 1961; Midgley and McCarthy, 1962; Levinthal *et al.,* 1962). It was once thought to be characterized by a size in the neighborhood of 14 S, but this has been proved erroneous (Monier *et al.,* 1962; Sagik *et al.,* 1962); the mRNA from a given organism may vary in size over a range of ca. 8 S to 45 S. Large messages are evidently polycistronic; that is, they are composite transcripts of several genes for polypeptide chains. The translation of a single polycistronic message into a number of separate polypeptide chains is shown directly in the case of RNA viral genomes that are single RNA molecules, are conserved throughout the latent period, and code for several proteins. The size of polyribosomal complexes indicates that polycistronic mRNA is used as such in the cell rather than being broken down into separate gene transcripts.

In cell-free systems neither rRNA nor tRNA are used as message, and no real evidence exists that they can be so used under any circumstances.

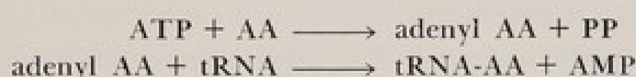
Several reasons can be advanced for their being nontranslatable, all of uncertain validity. Synthetic polyribonucleotides that tend to assume the helix configuration, e.g., those that have alternating complementary bases, show practically no message activity in comparison to those that have little secondary structure. Both tRNA and rRNA have a more extensive secondary structure than does mRNA, to judge by the criteria of melting curves and X-ray diffraction. The known yeast alanyl tRNA sequence is such that only a few small portions of the chain at a time can be in the helix form, but this might be enough to impede translation. It appears that the X-ray diffraction work attributing extensive helical structure to tRNA was actually done on partially degraded rRNA. It remains at least plausible that rRNA and tRNA are nontranslatable because of their secondary structure (Singer *et al.*, 1963). Another possibility is that specific terminal sequences may prevent attachment to ribosomes. Finally, the presence of unusual bases in specific positions may be a basis for nonfunctionality as message, particularly in the case of tRNA.

Whereas rRNA and tRNA are generally conserved, the mRNA in typical cases has a lifetime in the cell of only a few minutes. In *E. coli*, mRNA persists about 3 minutes, time enough to be translated about 10 times. Relatively stable examples of mRNA are known in mammalian systems, e.g., the hemoglobin mRNA in reticulocytes (Allen and Schweet, 1962) and in several RNA viral genomes. The mRNA for collagen, however, is unstable (Bekhor and Bavetta, 1965). The general stability of mRNA in liver has been reported (Revel and Hiatt, 1964). The instability of mRNA in bacteria is generally evident from turnover rates and from the very large fraction of total protein synthesis that is sensitive to fast inhibition by actinomycin D, which stops transcription but not translation (Reich *et al.*, 1961; Hurwitz *et al.*, 1962b; Goldberg *et al.*, 1962; Kahan *et al.*, 1963). In so far as stable mRNA is present, protein synthesis should continue in the presence of actinomycin D whereas all RNA synthesis is blocked except that which takes place by nontranscriptional means such as end addition (Preiss *et al.*, 1961; Starr and Goldthwaite, 1963; Anthony *et al.*, 1963; Daniel and Littauer, 1963), or on an RNA template. The intrinsic characteristics or surrounding circumstances that distinguish stable from unstable mRNA remain mysterious, as does the mechanism of breakdown. The initiation of breakdown probably renders the mRNA untranslatable since the products of partial translation expected to be formed during the breakdown process are nowhere evident. Mutants of *E. coli* that lack ribonuclease I have been isolated (Gesteland, 1965), and such mutants do not show prolonged message lifetime although

they grow somewhat more slowly than normal. The occurrence of the mutants indicates that ribonuclease is not essential to viability and that mRNA degradation must be attributed to some other enzyme or enzymes. A similar conclusion has been reached for mRNA degradation in a cell-free protein-synthesizing system (Barondes and Nirenberg, 1962).

The Translation Process

Translation can be naturally subdivided into two stages which may be called translation I and translation II. Translation I consists of the formation of aminoacyl tRNA in two steps, activation and transfer:



$$\text{ATP} + \text{AA} \longrightarrow \text{adenyl AA} + \text{PP}$$
$$\text{adenyl AA} + \text{tRNA} \longrightarrow \text{tRNA-AA} + \text{AMP}$$

Both are carried out by the same enzyme, the transfer enzyme, which is simultaneously specific for the amino acid and for an acceptor RNA that has the appropriate codon recognition specificity for its function in translation II. The interaction of tRNA and transfer enzymes shows species specificity, but surprisingly little in view of the phylogenetic remoteness of mixtures that still have partially normal function (Yamane and Sueoka, 1963).

Translation II comprises the events surrounding attachment of tRNA-AA to a message-ribosome combination. The combination can be visualized as having three sites per ribosome (Wettstein and Noll, 1965); first, an entrance site to which the charged tRNA is initially bound; second, a chain-attached site to which the charged tRNA is moved concomitantly with the formation of the peptide linkage between the amino group of its amino acid and the carboxyl end of the growing polypeptide chain, that is, the end formerly attached to the preceding tRNA; and third, an exit site temporarily occupied by the displaced uncharged tRNA. The uncharged tRNA is loosely bound to the exit site and is in equilibrium with uncharged tRNA in solution. In contrast, both the charged and the chain-attached tRNA are firmly bound to their sites, provided the ionic environment is favorable. They require GTP for release, and the entire complex possesses GTPase activity (Conway and Lipmann, 1964). The role of GTP in translation is not understood, although it has been suggested that GTP provides energy for the advancement of the ribosome along the message. On a message of normal length, several ribosomes, each with a growing polypeptide chain, progress simultaneously, separated from their neighbors by about 100 nucleotides, the polyribosomal complex (J. R. Warner *et al.*, 1963).

In addition to mRNA, ribosomes, charged tRNA, and Mg^{++}, transla-

tion II requires two supernatant protein fractions (Nakamoto *et al.*, 1963; Allende *et al.*, 1964; Bishop and Schweet, 1961; Fessenden and Moldave, 1961). It has been suggested that one of these is the true peptide polymerase and that the other is related in some way to the GTP requirement (Arlinghaus *et al.*, 1964). Until their separate functions are clear, they may be grouped together as peptide polymerase. Finally, the stable binding of charged tRNA to the mRNA-ribosome complex is dependent on the presence of NH_4^+ or K^+; of these, NH_4^+ is the most active (Conway, 1964). The stabilizing action of these cations is reversed by Li^+; hence, we have some reason to attribute the classic lithium effects on embryogenesis to inhibition of protein synthesis.

The correct specification of the amino acid at each position in the polypeptide chain depends on the accurate maintenance of a reading frame, since the code is nonoverlapping (Crick *et al.*, 1962). This means that the point of initiation of translation II is of critical importance in establishing the reading frame for the entire message. When the message is polycistronic, the problem of chain delineation arises in translation II just as it does in transcription. Signals for polypeptide chain termination must be present at appropriate points as part of the message itself. It seems likely that a very small number of codons, perhaps only one, is reserved for this purpose. The conjecture can be entertained that a codon that signifies chain termination has a corresponding tRNA.

At each event of translation II the codon-anticodon fit is sufficient to specify the amino acid for the local position. The question whether the amino acid itself plays any role in translation II has been answered negatively in one case at least, by means of an ingenious experiment by Chapeville *et al.* (1962). Charged cysteine tRNA was subjected to a catalytic reduction with Raney nickel, and the cysteine converted to alanine without otherwise altering the tRNA-AA. Normally, poly UC as synthetic message supports the incorporation of cysteine but not alanine into peptides, while poly UG supports alanine but not cysteine incorporation. After the conversion of cysteinyl to alanyl tRNA, however, alanine was incorporated with poly UC and not with poly UG. This indicates rather strongly that the role of the amino acid in specificity is confined to translation I.

Evidence Concerning the Genetic Basis of the Gene Action System

Neither fact nor convincing argument can be advanced against the surmise that the protein components of the gene action system are formed in the same way as the majority of the cell proteins. Conclusive evidence of this will be obtained when mutations in the genes for these proteins

are identified and mapped. With respect to the RNA components, the misleading circumstance that their base composition in many organisms does not resemble that of the DNA, provided grounds for the erroneous belief that they are not copied from DNA. Evidence for the existence of the *ur*-genes for rRNA and tRNA is available as a result of techniques for detection and measurement of molecular hybridization (Hall and Spiegelman, 1961; Bolton and McCarthy, 1962; Nygaard and Hall, 1963; Gillespie and Spiegelman, 1965). Under appropriate conditions, RNA and denatured (single-stranded) DNA will form a double helix, provided their sequences are complementary. The specific hybrid helical structure is very resistant to ribonuclease in comparison to the aggregates formed of noncomplementary RNA and DNA. Enzyme-resistant associations of radioactive labeled RNA with DNA can be identified in pycnographic fractions or adsorbed to nitrocellulose which adsorbs neither RNA nor its breakdown products. Addition of increasing amounts of RNA to a fixed amount of DNA leads to a saturation plateau for the RNA-DNA hybrid from which the proportion of DNA hybridizable with the given RNA follows directly.

By such a procedure, Yankofsky and Spiegelman (1962a), showed that 0.3% of the DNA of *E. coli* is complementary to rRNA. Discrepancies between the base compositions of RNA and DNA become entirely understandable when it is recognized that a very small fraction of the DNA is the template for the bulk of the RNA (rRNA makes up about 85% of the total). Since the molecular weight equivalent of the *E. coli* genome is about 3×10^9, the proportion of this, 0.003, that is complementary to rRNA is about 10^7 daltons. The combined 16 S and 23 S pieces weigh about 2×10^6, hence *E. coli* has about 5 genes for each size class of rRNA. Similar experiments in *Drosophila* (Ritossa and Spiegelman, 1965; Vermeulen and Atwood, 1965) indicated a redundancy of about 200-fold for the rRNA genes. By means of molecular hybridization with DNA of *Drosophila* stocks having different numbers of nucleolus organizer regions, Ritossa and Spiegelman have located the entire rRNA template complex in the nucleolus organizer region. Similarly, Vermeulen and Atwood (1965) have obtained a genetic map position for the rRNA gene complex in *E. coli*. In *Xenopus*, a remarkable anucleolate mutant was shown by Brown and Gurdon (1964) to synthesize no rRNA when homozygous; embryos survive to a tail bud stage on rRNA of maternal origin. Thus the mutant seems to be either a deletion or inactivation of the rRNA gene complex. Ritossa and Atwood (1965) have identified a *bobbed* mutation in *Drosophila melanogaster* as a partial deletion of the rRNA gene complex, since only about 0.1% of the DNA of this stock is

hybridizable to rRNA, whereas 0.3% is hybridizable in normal stocks. The phenotype of *bobbed*—delayed development, short bristles and etched tergites—may be attributed to a decrease in the maximum rate of protein synthesis resulting from a limited rate of rRNA production. In the uninucleolate heterozygote of *Xenopus,* rRNA synthesis is apparently regulated to produce the same amount as wild type, but if such regulation occurs in *Drosophila,* it is not sufficient to compensate for loss of a major portion of the rRNA genes.

Up to a certain point, the redundancy of rRNA *ur*-genes may be proportional to genome size as an adaptation to a requirement that the size of the gene action system be optimally adjusted to the number of functioning genes. This proportionality is seen in the comparison of *Drosophila* and *E. coli,* but evidence has been advanced that HeLa cells, with a much larger genome than *Drosophila,* have about the same redundancy of rRNA genes (McConkey and Hopkins, 1964). A high degree of redundancy implies that mutations in the complex would create relatively little selective differential; hence, the question arises of how a highly redundant complex can remain homogeneous in the face of mutation pressure. Some have favored the hypothesis that the apparent redundancy results from the elaboration of "nongenetic DNA" from a gene that is single when passed to successive generations. Actually, the complex may be as heterogeneous as selection will permit, and may multiply or eliminate parts by means of unequal crossing-over. Concerning the question of orderly heterogeneity of the type required for the production of specifically different types of ribosomes, no convincing evidence for or against it has been advanced. The most that can be said is that the number of different kinds of rRNA genes that are indispensable is less than the total number of copies; otherwise, viable partial deletions could not occur. Experiments on competition of the two size classes for hybridization sites show that they do not compete, hence their sequences are different (Yankofsky and Spiegelman, 1962b, 1963). The arrangement of the different genes in the complex is not yet clear, but if the suggested 45S precursor of rRNA (Scherrer *et al.*, 1963; Perry *et al.*, 1964) should be substantiated it would be obvious that the genes for the two sizes alternate and are transcribed into a larger unit later cleaved at a specific point.

The tRNA genes have not yet been located genetically except in a negative or indirect sense. They are not in the nucleolus organizer region and it is possible that they are at the loci of general suppressor mutations that are interpretable on certain assumptions, as changes in the anticodon. The reported proportion of DNA hybridizable with

tRNA in *E. coli* (Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962) leaves little room for the redundancy which is a necessary prerequisite for suppressor mutations in the anticodons, but the value may be low for technical reasons. In *Drosophila,* if 60 kinds of tRNA are present; each would have a redundancy of about 15, based on the saturation plateau of 0.02% for hybridization with the total tRNA. Several reasons can be advanced in support of the conjecture that the dominant mutants known as *Minutes* are deletions of the tRNA loci: The number of different *Minutes* calculable from the available data is in the neighborhood of 60; *Minutes* are predominantly based on cytologically visible deletions, which would not be the case for mutations affecting nonredundant loci; the phenotype resembles that of *bobbed,* and could plausibly be attributed to a general retardation of protein synthesis.

Many indications suggest that *ur*-genes are themselves subject to regulation. For example, the transplantation of a nucleus actively synthesizing rRNA to an enucleated egg results in cessation of rRNA synthesis in that nucleus and in its descendants until the stage of development is reached when synthesis would normally begin (Gurdon and Brown, 1965). Another example of *ur*-gene regulation is the inhibition of RNA synthesis in normal strains of *E. coli* by prevention of protein synthesis, e.g., amino acid deprivation in auxotrophs. This regulatory mechanism is absent in the so-called "relaxed" mutants which continue to make RNA when protein synthesis is inhibited. A gene in which mutations to "relaxed" occur has been mapped by Stent and Brenner (1961). These examples are especially significant because the regulated process is so easily identified; the regulation of nontranslatable RNA components must, of course, occur at the transcription level.

Specific Alteration of Nucleic Acids

The tRNA, DNA, and possibly to a lesser extent the rRNA, contain a variety of unusual nucleotides as minor components (Littlefield and Dunn, 1958; Smith and Dunn, 1959; Berquist and Matthews, 1962). Some of these have substituted bases; e.g., thymine (unusual in RNA); 2-methyladenine; 6-methylaminopurine; 6-dimethylaminopurine; 1-methylguanine; 6-hydroxy-2-methylaminopurine; and 6-hydroxy-2-methyldiaminopurine. Two others, pseudouridine (Cohn, 1959) and neoguanylate (Hemmens, 1963), have the pentose attached at an unusual position on the base. If such minor constituents entered the chain during polymerization as analogues of regular bases, one would expect a regular base to predominate at any given position, the occurrence of the rare analogue being limited by its frequency in the nucleotide pool. On the other hand,

if minor nucleotides *regularly* occupy specific positions in polynucleotide chains, it is reasonable to suppose that they represent specific alterations that occur after rather than during polymerization.

It is now clear that the positions of the minor nucleotides are usually —perhaps always—precisely determined. The species specificity of the alterations (Srinivasan and Borek, 1963), and the complete structure of yeast alanyl tRNA (Holley *et al.,* 1965), give evidence of their unique positional specificity. Enzymatic methylation of preformed nucleic acids (Mandel and Borek, 1961, 1963; Borek *et al.,* 1962; Fleissner and Borek, 1962, 1963; Gold and Hurwitz, 1963) has been repeatedly demonstrated; e.g., *E. coli* yields at least five methylating enzymes, each for a different base methylation (Gold *et al.,* 1963). The evidence for species specificity is that RNA exhaustively methylated by the enzymes of its own species still has positions that can be methylated by the enzymes of other species, and vice versa. The enzymes involved in the formation of pseudouridine and neoguanylate are unknown, but they are interesting because the reaction—removal of the base followed by reattachment at a different position on the ring—seems hard to accomplish.

The functional significance of altered nucleic acids is not fully understood. The source of methyl groups is methionine (Biswas *et al.,* 1961); hence, a "methyl-poor" tRNA is produced in methionine-deprived methionine auxotrophs of the relaxed strains of *E. coli* (strains that continue RNA synthesis during amino acid deprivation). At least some kinds of tRNA can be specifically charged with their amino acid while in the methyl-poor condition (J. L. Starr, 1963a), but the experiments are not extensive enough to conclude with confidence that entirely normal translation would occur without prior methylation of the tRNA (J. L. Starr, 1963b). Evidence has been presented that *E. coli* transfer enzymes will charge methyl-poor tRNA, whereas yeast transfer enzymes will not. In reference to the same point, species specificity of methylation has been proven, yet at least some widely heterologous enzyme-RNA combinations can translate *in vitro.* The evidence, so far as it goes, suggests that the pattern of methylation is generally not a decisive determinant of functional specificity of RNA in translation.

A known function of specific base alterations is to provide distinguishing characteristics that enable autogenous DNA to escape enzymatic degradation while DNA from outside sources is destroyed. The effect is seen as a host-controlled modification of viral DNA (Arber and Dussoix, 1962). When the bacteriophage infects the same host strain on which it was grown, a high proportion of the infected cells produce plaques, whereas the plating efficiency is extremely low if the phage has been

grown in certain strains differing from the one in which it is assayed. This reaction has been shown to involve a dual system among host strains; a given strain has a specific DNA methylation pattern and a corresponding DNA-degrading system that exempts DNA with the homologous pattern of methylation (Dussoix and Arber, 1965; Arber, 1965). It is not certain whether the methylating enzymes and nucleases alone are sufficient to establish and recognize the modification. The significant point is that superimposed modifications of nucleic acids have at least the potential for playing a role in the regulation of gene action, although no evidence for such a role has yet been advanced.

The Regulation of Gene Function

Superimposed on the gene action system, mechanisms have evolved whereby the transcription or translation of a gene is made conditional upon a specific stimulus. Most of the experimental evidence pertaining to such mechanisms has been obtained in microorganisms that do not have developmental patterns of the kinds we are most anxious to explain; hence, the question whether the metazoa and metaphyta have evolved any means of regulation of gene function different in principle from those that have been elucidated in microbial systems remains open. It is extremely unlikely, however, that regulatory mechanisms will be found that could not have been anticipated on the basis of present knowledge of the gene action system and the current generalized model for its control.

The Jacob-Monod model for gene regulation (Jacob and Monod, 1961) derives from genetic evidence related to systems in which exogenous effector substances control the synthesis of specific enzymes. In such a system, genetic mapping of mutations that disturb the response to the effector reveals the presence of a regulatory gene (or genes) at a location distinct from the location of the gene for the enzyme for which synthesis is being regulated. The product of the regulatory gene, formed by the normal processes of transcription and translation, interacts specifically with both the effector substance and the regulated gene. A majority of mutations in the regulatory gene result in the regulated gene remaining active irrespective of the presence or absence of effector, and irrespective of whether the normal action of the effector is to activate or to repress the regulated gene. Mutations of this type are recessive to the wild type in merozygotes (Pardee *et al.*, 1959); hence, the regulatory gene product is always a repressor, although in some cases it is active only in the presence of effector and in other cases only in the absence of effector.

The locus of interaction with the repressor in the regulated gene or

gene product is known as the operator. The existence of the operator, defined in this way, is not a matter of dispute. The operator may also be defined as a map region, within or adjacent to the regulated gene, of a group of mutations that prevent repression but are not recessive to the wild-type allele in a merozygote. Thus, according to the model, the repressor has two different kinds of sites, one specific for the operator and one specific for the effector. Finally, the repressor or complex is so constructed that the binding of effector to the effector site changes the configuration of the operator site; ample precedent for this mechanism is found among enzymes subject to feedback inhibition by end products structurally unrelated to their substrates. The model predicts other types of mutations, i.e., one that makes the repressor unable to respond to effector, thus causing dominant repression; and one present in the operator that prevents gene action. These mutations have been found, but the interpretation of mutations of the latter type and the fine structure of the operator region remain in doubt.

Ordinarily, more than one gene is subject to the same regulation. A gene ensemble that is coordinately repressed may be an operon transcribed together as one polycistronic message, or a group of separately transcribed genes having operators with the same specificity, the regulon (Maas and Clark, 1964). In either case it is a relevant question whether repression blocks transcription or translation. Two experiments have shown very clearly that the regulation of the *lac* and *gal* operons in *E. coli* is in fact a regulation of transcription (Attardi *et al.*, 1962; Hayashi *et al.*, 1963). Both are based on molecular hybridization of mRNA with DNA greatly enriched in the genes in question. Such DNA is obtained from bacteriophage strains that have incorporated a small portion of bacterial DNA into their genomes so that this portion is duplicated along with the viral DNA, or from the *E. coli lac* episome grown in *Serratia*. The DNA isolated from phages P1 *lac*, *λdg*, or *Serratia* F-*lac*, was hybridized with labeled RNA from *E. coli* with active or inactive *lac* or *gal* genes. About 50 times as much specific mRNA was present for an active gene as for an inactive one.

Evidence of regulation at translation II has been obtained from an RNA viral genome, itself a polycistronic message in which some parts are translated many times before the proteins specified by other parts appear at all (Ohtaka and Spiegelman, 1963). This phenomenon raises two separate questions: First, how can the point of translation regularly pass over a particular part of the message more than once without traversing the remainder of the message; and second, what is the stimulus that initiates formation of the late proteins? These questions are not settled,

and the generality of control at this level is also uncertain. This example is clearly distinct from the well-known polarity mutants that have been interpreted as partial blockades of translation (Ames and Hartman, 1963). Such mutations result in decreased production of enzymes specified by genes located between the mutation and the distal end of the operon, but normal amounts of enzymes specified by genes between the operator end and the mutation. The enzyme of the gene in which the mutation has occurred is typically inactive. It has been suggested that such mutations originate rare codons for which the corresponding tRNA is in short supply. This explanation is inadequate or incomplete, however, since a delay at a given point in translation cannot of itself alter the coordinate translation of the genes in an operon. An additional assumption is required, e.g., that premature chain termination occurs with a certain frequency.

Every hypothesis involving translation blockade has an implication that has not been given sufficient attention, namely, that the relation between the directions of transcription and translation is fixed. Let us assume that transcription proceeds from the operator to the distal end of the operon. On that assumption, in order for a polarity mutant to be a translation blockade, the translation of the message must proceed in the same direction as the transcription of the operon. It is known that mRNA is synthesized so that the 3′-hydroxyl end is last to be added *in vitro* (Bremer *et al.,* 1965) and in intact *E. coli* (Goldstein *et al.,* 1965). Translation proceeds from the N-terminal to the C-terminal amino acid (Nathans, 1964; Naughton and Dintzis, 1962; Goldstein and Brown, 1961; Bishop *et al.,* 1960); hence the direction of translation with respect to the sense of the mRNA can be determined by establishing a correspondence between terminal codons and terminal amino acids. The most direct evidence is the *in vitro* translation of the hexanucleotide AAAUUU into the lys-phe dipeptide (Thach *et al.,* 1965), and the translation of a polynucleotide of the form A_nC into polylysine with a C-terminal asparagine (Salas *et al.,* 1965). These experiments show that the direction of translation is indeed the same as that of transcription. Some contradictory evidence of a less direct nature remains to be explained (Eikenberry and Rich, 1965; Williamson and Schweet, 1965; Cramer *et al.,* 1964; Michelson and Grunberg-Manago, 1964). The weight of evidence is thus consistent with the standard hypothesis concerning polarity mutants. The interesting conjectures of Ames and Hartman based on their data on polarity mutants were elaborated by Stent (1964) into a general hypothesis of gene control at the translation level. Although the hypothesis is possible in a formal sense, it is not supported by available data pointing to transcrip-

tion as the controlled process, and it predicts contrary to experience, that inappropriate control or "modulating codons" would arise rather frequently by mutation.

Concerning the question whether a generalization of the Jacob-Monod model will be sufficient to explain development, detailed analogies between microbial and ontogenetic systems remain to be drawn. The effector substances that would ostensibly act during embryogenesis are of endogenous origin and are uncharacterized. The morphogenetic and biochemical levels are not yet connected by an unbroken chain of causation. Bacteria do not have histones, but it may be that the presence of histones in more complex organisms has diverted attention from highly specific regulatory proteins that have no nonspecific affinity for nucleic acids, and whose interactions with the genes have therefore remained obscure. We can maintain with confidence, however, that the key mechanisms of cellular differentiation will be found to act through the processes and components of the gene action system; hence, the ubiquitous features of this system are a guide to experimentation and to the restriction of hypotheses to the domain of the worthwhile.

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Structural Basis of the Specificity of Antibodies

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Introduction

Investigations initiated by the work of Edelman (1959) have shown that antibody molecules are made up of multiple polypeptide subunits linked by disulfide bonds and weak noncovalent interactions (Edelman and Poulik, 1961; Fleischman *et al.*, 1962, 1963; Olins and Edelman, 1962). Methods have been devised for separating the component chains by reduction of disulfide bonds and subsequent exposure to conditions known to disrupt noncovalent interactions in proteins. The dissociation into subunits is reversible under appropriate conditions, and molecules similar to the native protein in configuration and molecular weight can be reconstituted *in vitro*. If the immunoglobulin preparation has antibody activity, a significant proportion of the activity may be restored during the reassociation of subunits. Such investigations have permitted certain conclusions regarding the basis of antibody specificity, and one purpose of this paper is to summarize pertinent data. We will also discuss some recent structural studies and briefly review the accumulating evidence indicating that differences in antibody specificity are based on variation in amino acid sequence.

Antibody activity is associated with molecules collectively designated immunoglobulins. Table I summarizes some of the properties of three major classes common to many vertebrate species. In the guinea pig and in the mouse there are two 7 S immunoglobulins, distinct from γA, which have been designated γ_1 and γ_2 (Benacerraf *et al.*, 1963; Nussenzweig *et al.*, 1964; Fahey *et al.*, 1964). The former, which migrates more rapidly toward the anode during electrophoresis at neutral pH, mediates cutaneous anaphylactic reactions within the same species but does not fix guinea pig complement. The opposite is true, with respect to both properties, of the γ_2-globulin. Guinea pig γ_1- and γ_2-globulins are similar with respect to carbohydrate content (Oettgen *et al.*, 1965), as well as sedimenta-

tion coefficient, and it is therefore difficult at present to decide which, if either, is the counterpart of the γG-globulins of other species.

Each of the three classes of molecule appears to comprise two types of polypeptide chains; the "light" chain has a molecular weight of approximately 20,000 (Edelman and Poulik, 1961; Pain, 1963; Small *et al.*, 1963) and is common to all three classes (Cohen, 1963; Carbonara and Heremans, 1963); the classes differ with respect to their "heavy" chains which have molecular weights of 50,000–55,000 in γG-globulin (Pain, 1963; Small *et al.*, 1963). A molecule of γG-globulin consists of two light and two heavy chains (Fleischman *et al.*, 1962, 1963; Pain, 1963). Bence Jones

TABLE I

PROPERTIES OF THE MAJOR CLASSES OF IMMUNOGLOBULIN[a, b]

Immune globulin	Sedimentation coefficient	Molecular weight	Carbohydrate content	Dimensions
$\gamma G = \gamma_2$[c]	6.5 S	150,000	2–3%	35 × 280 Å
γA	6.5 S + polymers	150,000 + polymers	10%	?
γM[d]	19 S	800,000	10%	?

[a] The three classes have antigenic determinants in common present on "light" polypeptide chains (molecular weight, 20,000).

[b] Antibody activity is associated with all three classes.

[c] Two classes similar to γG, designated γ_1 and γ_2, have been demonstrated in guinea pigs and mice (see text).

[d] γM is depolymerized from 19 S to 7.5 S by mild reduction.

proteins, which are secreted in the urine of patients with multiple myeloma, are monomers or dimers of light polypeptide chains (Edelman and Gally, 1962). Incomplete heavy chains[1] have been found in the urine of a few persons with lymphatic malignancies (Franklin *et al.*, 1964; Osserman and Takatsuki, 1964). Such incomplete naturally occurring molecules are ordinarily included in the category of immunoglobulins.

A given type of polypeptide chain is characterized by marked heterogeneity, even within a single immunoglobulin class of a given species. It seems likely, for example, that antibody specificity is determined by the amino acid sequence of both light and heavy chains. If this is correct, the variety of sequences in at least a portion of each type of polypeptide chain is obviously very large. Another basis of heterogeneity has been elucidated through studies of the antigenic determinants of the poly-

[1] The protein secreted corresponds closely in structure to the inactive fragment (Fc) of a papain digest.

peptide chains of immunoglobulins. For example, the light and heavy chains of rabbit γG-immunoglobulin are under the control of at least two genetic loci, with three alleles at each locus. The phenotypic expression is an antigenic determinant, or group of determinants on the chain, which can evoke antibody formation on injection into a rabbit lacking the determinants. The classes of antigenic determinants are called allotypes (Oudin, 1956). An individual rabbit thus may possess light chains with one or two different allotypic specificities encompassed within the light chains of its γG-globulin, and one or two within the heavy chains. A single molecule, however, possesses only one type of light chain allotypic determinant and one type of heavy chain determinant (Oudin, 1961; Dray and Nisonoff, 1963). The inheritance of allotypic specificities is Mendelian and is not sex-linked. Each genotype is expressed phenotypically.

Variability of polypeptide chains similarly under genetic control and similarly characterized phenotypically by antigenic determinants on the chains, is also present in human immunoglobulins. The determinants on the heavy and light chains are known as the Gm and Inv groups, respectively (Grubb and Laurell, 1956; Ropartz *et al.*, 1961). The Gm groups in particular are complex (Fudenberg, 1963) and will not be discussed here.

Another source of variability among polypeptide chains is exemplified by the existence of two subgroups, distinct from Inv groups, of the light chains of human immunoglobulins (Korngold and Lipari, 1956a,b; Burtin *et al.*, 1956). Again, these subgroups are characterized by antigenic determinants present in each of the three major classes of immunoglobulin. Appropriate antisera can be prepared in rabbits. In contrast to rabbit allotypes, and to human Gm and Inv groups, both of these subgroups are represented in the sera of all normal individuals (Franklin, 1962; Mannik and Kunkel, 1963; Migita and Putnam, 1963; Fahey, 1963). Group K (or I) comprises about 60% and Group L (or II), 30% of the population of normal γG-immunoglobulin molecules (Mannik and Kunkel, 1963). Subgroups of normal populations of human heavy chains, characterized by their antigenic properties, have also been described (Dray, 1960; Lichter and Dray, 1964; Grey and Kunkel, 1964; Terry and Fahey, 1964; Ballieux *et al.*, 1964).

In view of these manifold sources of heterogeneity, one may reasonably ask what characteristics are common to the light chains and heavy chains of a given class of immunoglobulin in a particular species. A definitive answer to this question will require a large amount of data on amino acid sequences in the various subgroups. At present, one can say that light

chains of a particular class of immunoglobulin have in common the ability of combine stoichiometrically and spontaneously with heavy chains to form a complete molecule of the correct molecular size. This implies there is at least one region on each light polypeptide chain complementary to a region on a heavy chain (see Fig. 1), and indicates one type of structural invariance. In the rabbit, light chains of different allotypic specificity have some antigenic determinants in common, and *average* amino acid compositions as well as peptide maps (fingerprints) are very similar although not identical (Reisfeld *et al.*, 1965; Small *et al.*, 1965). (Fingerprints may fail to demonstrate the presence of variable segments of molecules or subunits because of the low yield of peptides derived from such segments.) Heavy chains of different allotypic specificity, as well as light chains, possess one or more similar antigenic determinants.

Thus there appear to be invariant as well as variable regions in the antibody molecule. Variations within a given class of polypeptide chain may reflect antibody specificity, heritable differences in sequence not associated with specificity, and the presence of multiple structural genes present in all individuals. The light chain groups, K and L, in the human immunoglobulins are in a somewhat special category since they share no antigenic determinants and yield fingerprints which appear to be unrelated (Putnam, 1962). They are, however, similar in molecular weight and in their capacity to form active antibody molecules in combination with heavy chains. The fact that the peptide maps are completely different does not exclude the possibility that many corresponding positions in the sequences of the two polypeptides may be occupied by the same amino acid.

Of the three major classes of complete immunoglobulin molecules (γG, γA, γM), the structure of the γG type is known in the greatest detail. A model incorporating many of the known structural features is shown in Fig. 1; it is essentially equivalent to the one proposed by Fougereau and Edelman (1965) and applies to rabbit and human γG-globulins. Considerable evidence indicates that the γG-globulins of many other species have similar structures.

The presence of multiple polypeptide chains in the γG-globulin molecule was established by the work of Edelman and his collaborators (Edelman, 1959; Edelman and Poulik, 1961). Subsequent to the reduction of interchain disulfide bonds and alkylation to prevent reoxidation, the polypeptide subunits can be separated by treatment with urea, 1 *M* propionic acid, guanidine hydrochloride, or detergent (Edelman and Poulik, 1961; Fleischman *et al.*, 1962; Small *et al.*, 1963; Marler *et al.*, 1964; Utsumi and Karush, 1964; Criddle, 1964). The light and heavy

chains can be isolated for analytical purposes on starch gel (Edelman and Poulik, 1961) or acrylamide gel (Cohen and Porter, 1964) in a dissociating solvent such as aqueous urea. On a preparative scale, the chains may be separated by gel filtration on Sephadex equilibrated with 1 *M* propionic acid (Fleischman *et al.*, 1962) or, at neutral pH, with sodium decyl sulfate (Utsumi and Karush, 1964). The heavy and light chains are eluted as successive peaks in approximately a 5:2 weight ratio, which is also the

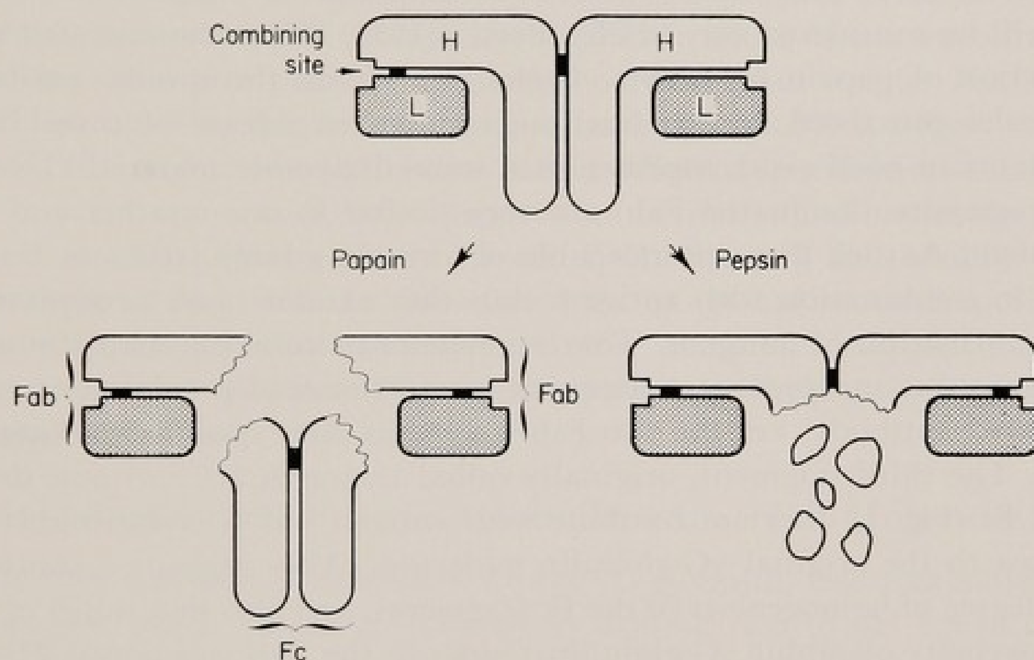

FIG. 1. Structural model of rabbit or human 7 S γG-immunoglobulin molecule, slightly modified from Fougereau and Edelman (1965). H and L signify "heavy" and "light" polypeptide chains of molecular weight 55,000 and 20,000, respectively. Each small dark rectangular area between chains represents an interchain disulfide bond. (In addition, there are about 17 intrachain disulfide bonds.) The H and L chains and the two halves of the Fc fragments are held together by noncovalent interactions as well as by a disulfide bond. Molecular weights of the Fab and Fc fragments are approximately 43,000 and 55,000, respectively. The size of a combining site is exaggerated, since it actually represents roughly 1–2% of the surface area of the molecule.

ratio of the molecular weights of the chains. Gel filtration in 1 *M* propionic acid has been used in most experiments in which biological activity is restored by reassociation of separated light and heavy chains, and these investigations are discussed in some detail later. This method of separation has also permitted accurate estimation of the molecular weights of the polypeptide subunits (Pain, 1963).

Comparison of antigenic determinants and amino acid compositions of polypeptide chains isolated by this method with those of the fragments

liberated by papain, has established the relationship between polypeptide chain structure and the fragments formed by enzymatic digestion shown in Fig. 1 (Fleischman *et al.*, 1962, 1963; Olins and Edelman, 1962).

Concurrently with the isolation and characterization of the subunits, useful information was derived from studies of the proteolysis of γG-globulin by enzymes such as papain and pepsin. The action of proteolytic enzymes on antibodies has been discussed in several reviews (e.g., Porter and Press, 1962; Eisen and Pearce, 1962; Nisonoff and Thorbecke, 1964) and will be considered very briefly. Porter (1958, 1959) demonstrated that the action of papain on rabbit γG-globulin, or on the specific antibody molecules contained in this fraction, resulted in release of three large fragments in good yield, together with some dialyzable material. Two of the fragments (designated Fab) are very similar to one another and are univalent. As such they are incapable of forming a large lattice or framework in combination with antigen; thus they cannot cause precipitation or agglutination of antigens. The fragments can combine with the antigen, however, and prevent subsequent interaction and precipitation with untreated antibody; i.e., the two Fab fragments have specific blocking activity. The third fragment, originally called fragment III and now designated Fc (Fig. 1) does not combine with antigen but is crystallizable, in contrast to the original γG-globulin molecules. This suggests a considerable degree of homogeneity of the Fc fragments, and also that much of the heterogeneity of rabbit γG-globulin resides in the Fab fragments. This is not surprising since the latter contain the combining sites which are necessarily variable. Certain biological activities, such as fixation to skin in passive cutaneous anaphylaxis and ability to cross the placenta in certain species, are associated with the inactive fragment. It may also be directly involved in complement fixation although this is controversial. In some instances, a large proportion of the antibodies formed on injection of γG-globulin into a heterologous species is directed against the Fc fragment (Porter, 1958, 1959). These subjects are considered in detail in the reviews cited above.

The action of pepsin on rabbit γG-globulin results in removal of a fragment that corresponds roughly to papain fragment Fc but is evidently somewhat smaller (Nisonoff *et al.*, 1960; Nisonoff and Hong, 1964; Nisonoff and Dixon, 1964; Jaquet and Cebra, 1965). After treatment with pepsin, the two fragments containing the active sites are linked by a disulfide bond. Thus, the major product of peptic digestion is still bivalent and capable of forming specific precipitates. It has a molecular weight approximately two-thirds that of the undegraded molecule. The

bivalent fragment formed by peptic digestion can be dissociated into two univalent fragments approximately equal in size, by reduction of one disulfide bond (Nisonoff *et al.*, 1961); see Fig. 1. There appear to be no additional noncovalent interactions linking the univalent fragments at this juncture, since separation of the two fragments occurs at neutral pH after reduction.

A considerable degree of recombination occurs through reoxidation, if the reducing agent is rapidly removed and the protein solution allowed to stand at neutral pH (Mandy *et al.*, 1961). Thus, the transformation from bivalent precipitating fragments of molecular weight 100,000 to univalent blocking fragments of molecular weight 50,000 is largely reversible. If, after reduction, antibodies of two different specificities are mixed, the product contains a large proportion of individual molecules with mixed specificity (Nisonoff and Rivers, 1961; Nisonoff and Mandy, 1962; Fudenberg *et al.*, 1964). In the case of the γG-globulins, such molecules do not appear to occur naturally. A more complete description of the effects of pepsin and reducing agent on rabbit γG-globulin is given by Nisonoff and Hong (1964).

Dissociation of Rabbit γG-Globulin into Half-Molecules

Another type of dissociation of rabbit γG-globulin, into half-molecules, occurs after mild reduction followed by acidification to pH 2.5 in dilute salt solution (Palmer *et al.*, 1963). Two factors favor the formation of half-molecules rather than separate chains. First is the greater lability of the disulfide bond linking the heavy chains (cf., Fig. 1) in comparison to that of the bonds joining light and heavy chains (Palmer *et al.*, 1963; Palmer and Nisonoff, 1964). Second, at low pH the noncovalent interactions joining the heavy chains are less stable than those between light and heavy chains. After reduction of all the interchain disulfide bonds, dissociation into half-molecules takes place under milder conditions than are required for separation of light from heavy chains (Hong and Nisonoff, 1965). Figure 2 compares the effects of pH on the sedimentation patterns of unreduced and reduced rabbit γG-globulin. At pH 3.3, the sedimentation coefficients are approximately the same; in contrast, at pH 2.5 that of reduced γG-globulin is only 70% as great ($s_{20,w} = 3.3$ versus 4.8). At the intermediate pH values of 2.9 and 2.7, two components are present in the reduced preparation; the slower component increases in amount as the pH is lowered. Unreduced γG-globulin exhibited a single peak at each pH investigated; the decrease in sedimentation coefficient is probably attributable to expansion of the molecule at low pH.

The 3.3 S component observed in Fig. 2 was found to have weight-average and Z-average molecular weights of 81,800 and 85,500, respectively (Palmer *et al.*, 1963). These values are consistent with a molecular weight of 75,000 and the presence of about 10% of undissociated γG-globulin (molecular weight, 150,000). A small amount of faster-sedimenting material was present in the preparation examined even after passage through Sephadex G-200. In later studies it was found that dissociation at pH 2.5

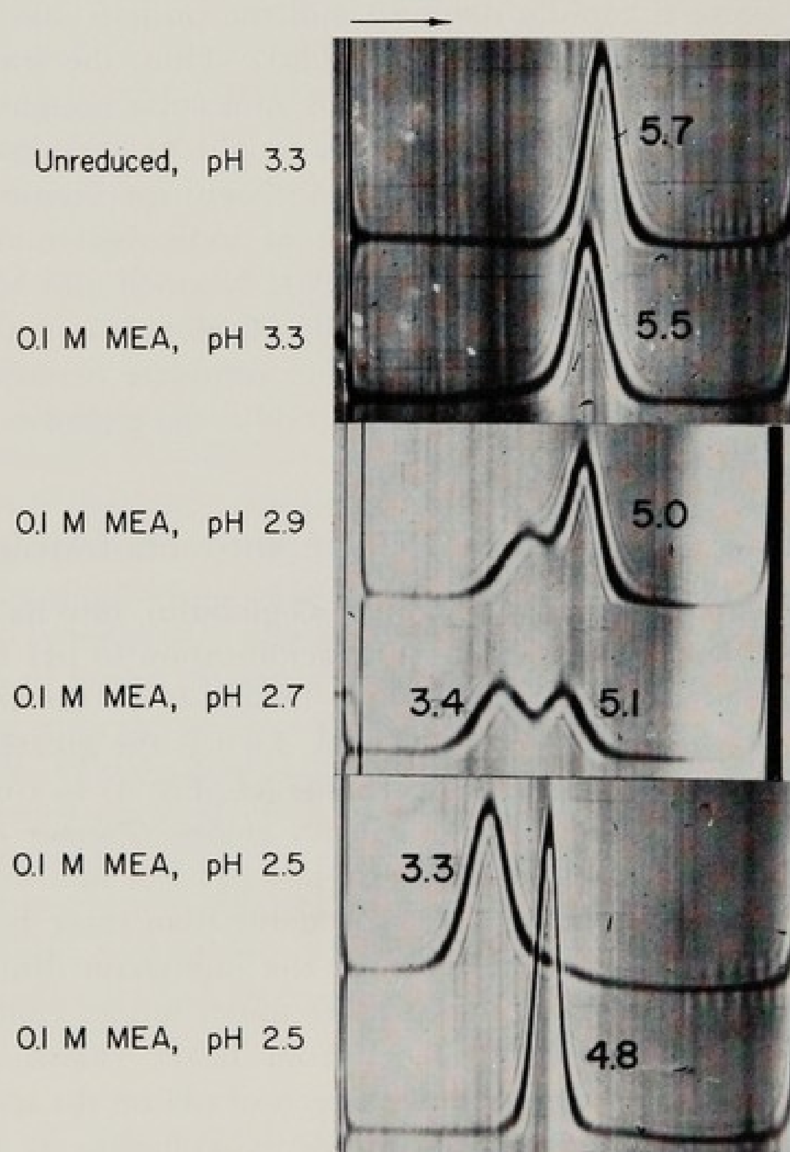

FIG. 2. Schlieren patterns of reduced and unreduced rabbit γG-globulin photographed after ultracentrifugation for 80 minutes at 59,780 rpm at 20°C (except G, 64 minutes). MEA is an abbreviation for 2-mercaptoethylamine, the reducing agent employed. The slower-moving, 3.3 S component consists of half-molecules of γG-globulin and appears in increasing amounts as the pH of the reduced protein is lowered from 3.3 to 2.5; the numerals are $s_{20,w}$ values; sedimentation is from left to right. (Palmer *et al.*, 1963.)

is more nearly complete in 0.025 *M* NaCl than in 0.1 *M* NaCl (Palmer and Nisonoff, 1964).

The data on molecular weights are therefore consistent with dissociation into half-molecules. Further evidence includes the symmetry of the schlieren peak at low pH and the fact that essentially complete dissociation occurs after a mild reduction that permits the separation of only small amounts of light chains in 1 *M* propionic acid. In addition, an experiment was carried out in which the extent of reduction was such that dissociation occurred in only a fraction of the population of molecules. After separation of the dissociable 3 S molecules from the undissociable (unreduced) molecules by gel filtration at pH 2.4, it was found that their amino acid compositions were the same within experimental error. This result is again consistent with a separation into symmetrical half-molecules.

Finally, a large proportion of the γG-globulin can be dissociated into half-molecules after reduction of slightly more than one disulfide bond for each molecule rendered dissociable (Palmer and Nisonoff, 1964). This supports the view that two subunits are separated and, together with the data on molecular weights, provides strong evidence for symmetrical cleavage into half-molecules.

Spontaneous Reassociation of Half-Molecule Subunits at Neutral pH

After dissociation into half-molecules at low pH, a large proportion of the γG-globulin spontaneously recombines at neutrality to form molecules having the same sedimentation coefficient as unreduced γG-globulin (~6 S). The recombination involves noncovalent interactions since it takes place even after sulfhydryl groups released on reduction have been inactivated by treatment with a reagent such as iodoacetate to prevent reoxidation. Schlieren patterns of dissociated and recombined preparations are shown in Fig. 3.

Physical chemical studies were carried out on a recombined preparation that had been purified by gel filtration (Fig. 3D). The product migrated as a symmetrical 6.3 S peak in the ultracentrifuge. Its molecular weight and diffusion constant agreed closely with corresponding values for unreduced γG-globulin. In another similar experiment the specific viscosities of native and reconstituted molecules were also found to agree within experimental error (Nisonoff and Hong, 1964).

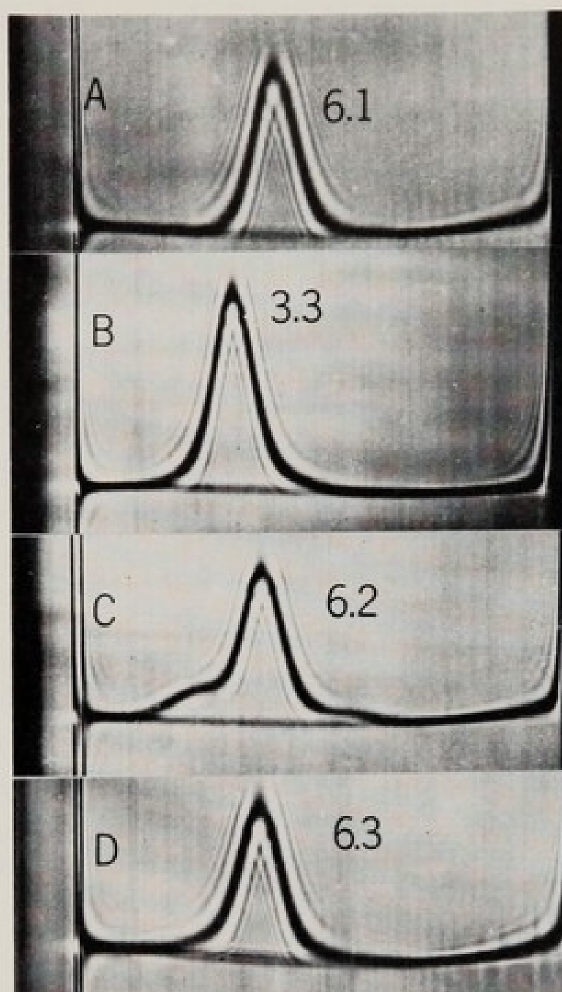

FIG. 3. Schlieren patterns obtained in the ultracentrifuge; an illustration of dissociation of reduced rabbit γG-globulin into half-molecules at low pH and reassociation at neutrality. Sedimentation is from left to right. A, Normal γG-globulin; B, γG-globulin reduced at pH 5 with 2-mercaptoethylamine, and then acidified to pH 2.4 in 0.035 *M* NaCl; C, above sample after neutralization to pH 8 by dialysis; D, above neutralized sample after filtration through Sephadex G-200 for isolation of the major component. Centrifugation at 59,780 rpm at 20°C (except B, 10°C). Photographs were taken after 48 minutes (except B, 80 minutes) at full speed. The numerals are $s_{20,w}$ values; Solvent for A, C, and D was saline-borate buffer, pH 8, ionic strength 0.16. (Nisonoff and Hong, 1964.)

Evidence for Univalence of Half-Molecules of Rabbit γG-Globulin

Since a half-molecule comprises a light and a heavy chain one would predict that it is univalent. A direct demonstration of this property is complicated by the spontaneous recombination of half-molecules at neutral pH at which antibody assays are carried out. An indirect approach took advantage of the finding that recombination of half-molecules of

purified antiovalbumin with the half-molecules of nonspecific γG-globulin is essentially random (Nisonoff and Palmer, 1964). Half-molecules of purified antiovalbumin were therefore recombined in the presence of an elevenfold excess of half-molecules of normal γG-globulin; the mixture was made at low pH and then neutralized (Hong *et al.,* 1965). On the assumption of random recombination, 11/12 of the half-molecules derived from antibody should have recombined with half-molecules of γG-globulin to form univalent 6 S molecules. It was found that the recombined mixed molecules were capable of specifically inhibiting the precipitation of untreated antiovalbumin with ovalbumin. This is consistent with univalence of the mixed 6 S molecules, and therefore of half-molecules of antiovalbumin. As a control in this experiment, half-molecules of purified antiovalbumin were allowed to recombine in the absence of normal γG-globulin. The product formed specific precipitates; 81% of the protein was precipitable by an optimal concentration of the antigen (Hong *et al.,* 1965).

Reversible Dissociation of Fragment Fc

As indicated earlier, the fact that two univalent (Fab′) fragments are linked only by a disulfide bond after peptic digestion suggests that most of the noncovalent interactions between heavy chains are present in fragment Fc and therefore involve the portions of the two heavy chains comprising this fragment (Fig. 1). One would thus expect that reduced fragment Fc might dissociate into half-fragments under conditions similar to those resulting in dissociation of reduced γG-globulin into half-molecules. Prior to our work on this problem, Marler *et al.* (1964) had shown that reduced fragment Fc dissociates in guanidine hydrochloride and that the molecular weight of the product (26,000) is half that of undissociated Fc. Our work (Inman and Nisonoff, 1965a,b) indicates that dissociation of reduced Fc can also take place under conditions very similar to those required for dissociation of reduced γG-globulin into half-molecules. Schlieren patterns illustrating the effect of pH on reduced Fc in 0.05 *M* NaCl are shown in Fig. 4; molecular weights are given in Table II. Dissociation occurs between pH 3.5 and 2.7, and increasing extents of dissociation are observable at intermediate pH values. The molecular weight of the dissociation products is approximately half that of undissociated Fc (Table II).

As in the case of reduced γG-globulin, dissociation of Fc is reversible at neutral pH; the neutralized product has a sedimentation coefficient and

molecular weight very close to those of undissociated Fc (Inman and Nisonoff, 1965a,b).

Other factors relevant to this dissociation may be summarized as follows: (a) Crystallized Fc prepared by digestion with papain in the presence of 0.001 *M* L-cysteine is only slightly dissociable at pH 2.4, whereas crystallized Fc prepared by digestion in the presence of 0.05 *M* L-cysteine

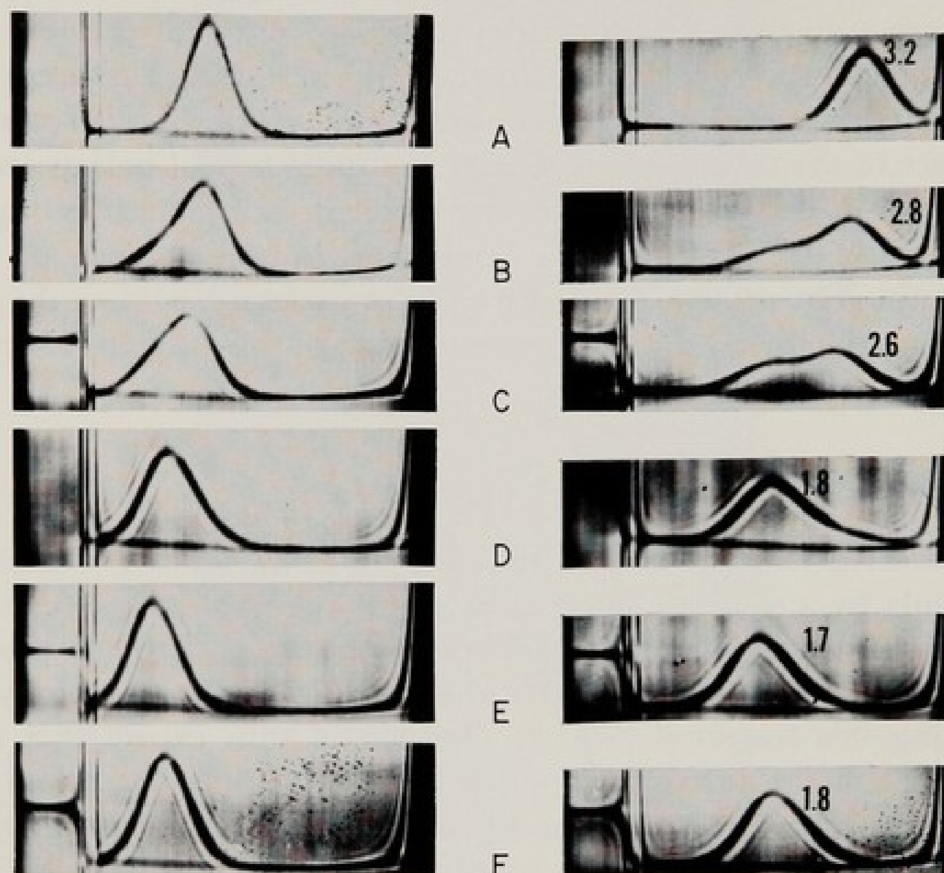

FIG. 4. The effect of pH on sedimentation of recrystallized Fc prepared by digestion with papain in the presence of 0.05 *M* L-cysteine. A–F correspond to pH values of 3.5, 3.2, 3.1, 3.0, 2.7, and 2.4, respectively; NaCl concentration, 0.05 *M*. Sedimentation (from left to right) for 80 minutes (photographs on the left) and 160 minutes (photographs on the right) at 59,780 rpm at 20°C. Numerals are $s_{20,w}$ values. The 1.8 S peak represents a half-fragment of Fc.

is about 90% dissociable; (b) Fc prepared with the lower concentration of L-cysteine is rendered dissociable by reduction with 0.05 *M* L-cysteine, thus indicating the presence of an interchain disulfide bond; (c) reduction of this interchain bond is reversible. The reduced Fc fragment loses its capacity for dissociation at low pH if reoxidation is permitted to occur after removal of the reducing agent. These results provide support for the molecular model in Fig. 1 and are consistent with the view that most

of the noncovalent interactions linking the heavy chains are present in fragment Fc. They also suggest that papain and pepsin cleave on opposite "sides" of the interchain disulfide bond joining the heavy chains (Fig. 1). After peptic digestion this bond links the two Fab′ fragments, whereas it

TABLE II
MOLECULAR WEIGHTS OF PURIFIED Fc[a]

Concentration of cysteine present during digestion (*M*)	Molecular weight[b]	pH[c]
0.001	48,800	3.5
0.001	41,500	2.4
0.05	52,900	3.5
0.05	24,100	2.4

[a] Prepared from rabbit γG-globulin by digestion with 2% by weight of papain in the presence of L-cysteine (Inman and Nisonoff, 1965a,b). Fragment Fc was purified by recrystallization or gel filtration.

[b] Molecular weights were determined by the method of sedimentation-diffusion.

[c] Refers to the pH during molecular weight determination.

is localized in Fc after papain digestion in the presence of a very low concentration of reducing agent.

In Vitro Complementation of Polypeptide Subunits of γG-Globulins

There is some uncertainty at present about the extent to which light and heavy polypeptide chains contribute to the active site of an antibody molecule. The problem is complicated by the difficulty (Feinstein *et al.*, 1963; Stemke, 1964) of preparing heavy chain fractions that are completely free of light chains. Certain tentative conclusions can be drawn from the limited data available:

(a) Isolated heavy chains usually have some antibody activity.

(b) Isolated light chains in general have little if any activity.

(c) Recombination of antibody heavy chains with antibody light chains results in an increase in activity over that of the heavy chains alone; such recombination occurs spontaneously through noncovalent interactions.

(d) Recombination of heavy chains of antibody with light chains derived from antibody of a different specificity, or from nonspecific γG-globulin, gives little enhancement, although molecules similar to native antibody in molecular weight are formed in good yield.

(e) Complementation is most effective when the light and heavy chains of a particular specificity are derived from the same animal.

(f) The presence of hapten stabilizes the noncovalent linkage of heavy and light chains. This results in the preference of heavy chains for homologous rather than heterologous light chains when competition is carried out *in vitro* in the presence of hapten.[2]

(g) The results of a number of investigations are characterized by considerable variation in the amounts of antibody activity associated with isolated heavy chains. The reasons for this variability have not been established. Factors that might be relevant are: (1) the specificity of the antibody; i.e., heavy chains of different antibodies might contribute to specificity to a variable extent; (2) the tendency of isolated heavy chains to aggregate; (3) differences in the extent of denaturation of chains during the isolation procedures; or, (4) the difficulty of completely separating heavy chains from light chains. In experiments in which the activity of isolated heavy chains was only a few per cent of that of the native antibody (cf. Table III), the possibility that this residual activity was actually attributable to contamination by light chains capable of complementation with the heavy chains has not been completely excluded.

Evidence for Antibody Activity in Isolated Heavy Chains

At least two investigations have indicated the association of a substantial amount of antibody activity with separated heavy chains. Fleischman *et al.* (1963), found that heavy chains of horse γG-globulin, containing antibody against rabbit γ-globulin, specifically coprecipitated when added to a mixture of the antigen and untreated antibody. The degree of coprecipitation of heavy chains was quantitatively similar to that observed on the addition of comparable amounts of the untreated, antibody-containing γG-globulin fraction to the same precipitating system. Specificity was indicated by the fact that the heavy chains did not coprecipitate when added to another horse antigen-antibody system. In addition, they found that heavy chains derived from a different horse antibody, antidiphtheria toxoid, specifically inhibited the homologous antigen-antibody reaction presumably through combination with antigen. The degree of inhibition per unit weight of added heavy chains was similar to that observed on the addition of untreated antibody globulin

[2] Such an experiment is performed by mixing a deficiency of heavy chains with the two types of light chain in 1 *M* propionic acid and bringing the mixture to neutral pH. The amount of each type of light chain bound to heavy chains is then determined.

to the precipitating mixture. Heavy chains derived from a different horse antibody had no effect.

Additional evidence for activity in the heavy chain fraction of γG-globulin comes from the studies of Utsumi and Karush (1964) who employed rabbit antibody specific for the azophenyl-β-lactoside hapten group. They dissociated the light and heavy chains of the reduced purified γG-antibody with 0.05 *M* sodium decyl sulfate at neutral pH; the chains were separated on Sephadex G-200 equilibrated with the detergent which was then removed on an ion-exchange column. Antigenic analysis indicated that the separation was essentially complete. Equilibrium dialysis was used to measure the capacity of the separated chains for binding hapten. More than half of the heavy chains were found to have active combining sites; the average binding constant, however, was about one-eighth that of reduced undissociated antibody. Expressed as unitary free energy of combination, the decrease in binding affinity was 13%. In view of the possibility that loss of activity was actually attributable to denaturation rather than to the removal of light chains, the investigators tentatively concluded that the binding activity was exclusively associated with heavy chains.

Antibody Activity of Recombined Heavy and Light Chains

Considerable evidence now indicates that the specific recombination of light and heavy chains may yield a product with more activity than that resulting from the combination of heavy chains of antibody with nonspecific light chains, or of antibody light chains with nonspecific heavy chains.[3] Suggestive evidence was first obtained by Franek and Nezlin (1963). After recombination of heavy and light chains derived from purified horse diphtheria antitoxin, about 3% of the protein was active, as judged by adsorption to an antigen covalently linked to cellulose which served as an inert matrix. Less than 1% of the protein was adsorbed when the heavy chains of diphtheria antitoxin were combined with light chains of a different antibody, or if light chains of the diphtheria antitoxin were combined with the heavy chains derived from a different horse antibody. Approximately 65% of the undegraded diphtheria antitoxin was specifically adsorbed. In the case of horse anti-tetanus toxin, however, there was little difference between the results obtained when the light chains were derived from the homologous anti-

[3] In the experiments to be discussed, mixtures of chains were made at low pH, usually in 1 *M* propionic acid, and then dialyzed against neutral buffer.

TABLE III

RECONSTITUTION OF ACTIVITY OF PURIFIED ANTIBODIES BY RECOMBINATION OF POLYPEPTIDE CHAINS[a]

Antibody[c]	Species	Reduced, alkylated, exposed to 1 *M* propionic acid	Percentage of original activity[b]					Assay method[e]	Reference
			H Chain[d]	L Chain	H + L	H + L_{het}[d]	L + H_{het}		
Anti-f1 phage	Guinea pig	3.6	0.4	< 0.1	1.0 (0.4)[f]	0.3 (0.4)[f]	< 0.1	A	Edelman *et al.* (1963)
Anti-f2 phage	Guinea pig	25	4.0	0.8	6.0 (2.0)	4.8 (2.0)	0.2	A	Edelman *et al.* (1963)
Antidinitrophenyl	Guinea pig	53	42	<22	64 (38)	—	—	B	Edelman *et al.* (1963)
Anti-f1 phage	Sheep	—	5.4	0.3	16 (3.9)	2.3	0.4	A	Fougereau *et al.* (1964)
Antibenzoate	Rabbit	58	—	—	51	6	2	B	Roholt *et al.* (1964)

[a] In each experiment mixtures of light and heavy chains in approximately equimolar amounts were prepared at low pH, and then brought to neutrality.

[b] Values in the table are percentages of the activity of the untreated, purified antibody.

[c] Antibodies were purified from the serum of an individual animal, except in the case of antibenzoate which was derived from a pool of sera.

[d] Abbreviations: H = heavy chains of the antibody; L = light chains of the antibody; L_{het} = light chains from a heterologous source, either nonspecific γG-globulin or a different antibody; H_{het} = heavy chains from a heterologous source.

[e] Assay methods: A, phage neutralization; B, hapten-binding capacity measured by equilibrium dialysis.

[f] Numbers in parentheses indicate activity expected if activities of the separate chains were additive.

body or from a different horse antibody. In either case the amount of protein adsorbed was less than 1%.

Thus with either antibody at least 97% of the activity was lost, possibly as a result of the fact that 6 *M* urea was used as solvent during the separation of chains on Sephadex. There would also appear to be some possibility of variation in the degree of nonspecific adsorption of the different preparations to cellulose.

Table III summarizes data which support the hypothesis that a specific combination of light and heavy chains from the same antibody is more effective in restoration of activity than a combination of specific heavy chains with nonspecific light chains or specific light chains with nonspecific heavy chains. The sixth column in Table III, labeled "H + L," compares the degree of activity observed on combination of specific heavy and light chains with that predicted from the activity of either type of chain measured separately; the predicted value is given in parentheses. In each case there is a definite enhancement attributable to interaction of the chains.

A comparison of the sixth and seventh columns indicates that light chains derived from the antibody are more effective in complementation of specific heavy chains than are light chains derived from a different antibody source or from normal γG-globulin.

The results in the eighth column (specific light chains + nonspecific heavy chains) indicate that the light chains of the antibodies investigated are relatively ineffective when combined with heterologous heavy chains.

The most striking results, obtained with rabbit antibenzoate antibody (Roholt *et al.*, 1964), are shown in the fifth row of Table III. Approximately 51% of the original activity, measured by the capacity for binding hapten, was restored on mixing the specific heavy and light chains. When chains from nonspecific γG-globulin were substituted for either specific heavy or specific light chains, nearly all of the activity was lost. The possibility was not eliminated that part of the binding by mixtures of specific and nonspecific chains, 6% and 2%, respectively, might have been due to the presence of a slight amount of residual heavy chain in the light chain fraction and vice versa.

An interesting effect of the presence of hapten during specific recombination was noted by Metzger and Singer (1963) who worked with rabbit antibody specific for the dinitrophenyl hapten group. This antibody retains the capacity for binding hapten at low pH. It was found that the yield of free light chains obtained on gel filtration of the reduced antibody in 1 *M* propionic acid was reduced from about 25% to 16% of the

total protein, when 6×10^{-6} *M* hapten, dinitrophenyllysine, was added to the reduced antibody preparation and to the buffer used for elution. This result shows that hapten stabilizes the noncovalent linkage of heavy and light chains thus decreasing the extent of dissociation in 1 *M* propionic acid.

In a second investigation (Metzger and Mannik, 1964), competition of specific and nonspecific light chains for specific heavy chains derived from rabbit antidinitrophenyl antibody was measured. Equal amounts of the two types of light chains were used, and the molar ratio of light to heavy chains was 2:1; i.e., light chains were present in excess. When the combination of light and heavy chains was carried out in the absence of hapten, the nonspecific and specific chains competed about equally well for combination with the specific heavy chains. However, when the mixture was made in the presence of hapten, 3×10^{-5} *M* dinitrophenylaminocaproic acid, the ratio of specific to nonspecific light chains in the final product, after neutralization, was about 2:1. This again indicates a specific stabilizing effect of hapten on the interaction of heavy and light chains of antibody. It was also noted that the amount of antibody activity restored on combination of specific light and heavy chains was increased when hapten was present during the association.

Recently, Roholt *et al.* (1965) reported that the mixing of heavy and light chains of antibenzoate antibody from the same rabbit resulted in good recovery of antibody activity. In contrast, when heavy chains of antibenzoate antibody of one rabbit were recombined with light chains of antibenzoate antibody from another rabbit there was little restoration of activity. A possible mechanistic explanation for this result might involve the geometry of the site; i.e., a site with a particular configuration may be formed by a variety of chain pairs, but the configuration of the heavy chains would dictate or at least restrict the range of structures of effective complementary light chains. Alternatively, interaction with the light chain might influence specificity by altering the configuration of a heavy chain with the interaction between chains taking place outside the active site. This possibility is based on the assumption that the heavy chain contains the site. Again, a variety of light and heavy chain combinations might produce an active site, but the light chains from one antibody molecule might not correctly influence the structure of the heavy chain from a different antibody molecule of the same specificity.

Evidence for Direct Participation of Light Chains in the Active Site of an Antibody

The enhancement of specific antibody activity by light chains is consistent with the possibility that a portion of the light chain is physically present in the active site of an antibody. As indicated, however, an alternative explanation is conceivable. The experiments of Metzger and Singer, cited above, demonstrating stabilization of the noncovalent interaction of heavy and light chains by hapten, are similarly consistent with direct participation of the light chain in the active site; stabilization by hapten could well result from a three-way interaction among hapten and the two types of polypeptide chain in the active site. As these workers indicated, however, the data do not exclude the possibility that hapten induces an alteration of configuration of the heavy chain which permits a stronger interaction with the light chain *outside* the active site.

Direct evidence in support of the physical participation of both types of chain in the active site was obtained by Metzger *et al.* (1964). Their previous work had shown that the active site of an antibody can be labeled by using specific hapten capable of interacting covalently with the side chains of proteins. For example, rabbit antibody against the dinitrophenyl hapten group can be specifically labeled with *p*-nitrophenyldiazonium fluoborate. The nitrophenyl portion of the molecule causes specific combination of the hapten with the antibody site. The diazonium group then reacts preferentially with a side chain in or very near the active site, by forming a covalent bond. Several lines of evidence have demonstrated the validity of this conclusion.

When antidinitrophenyl antibody was allowed to react with sufficient diazonium reagent so that 0.4 moles per mole of protein were incorporated, it was found that two-thirds of the covalently bound hapten was attached to heavy chains and one-third to light chains. This experiment provides strong evidence for the direct participation of both types of chain in the active site of at least part of the population of antibody molecules. In the same paper similar results were reported for another rabbit antibody specific for the azophenylarsonate hapten group.

Evidence That Differences in Specificity Are Associated with Differences in Amino Acid Sequence

Perhaps the most striking characteristic of antibodies that distinguishes them from other proteins is that an enormous variety of specificities is associated with relatively small differences in structure and amino acid

composition. Thus, there must be large sequences common to the various antibody molecules. Pauling's (1940) template theory took cognizance of this fact by proposing that the variety of specificities is associated with a single primary sequence and that specificity is determined during the folding of the polypeptide chain. Folding was assumed to occur in the presence of antigen which acts as a template and leaves an imprint on the completed antibody molecule.

The work of White (1961) and Anfinsen *et al.* (1961), and subsequent similar work with a number of enzymes, has demonstrated that after reduction of disulfide bonds and unfolding many proteins spontaneously regain their native structure if permitted to refold and reoxidize under appropriate conditions. This indicates that the final tertiary structure must be determined by the amino acid sequence. Such experiments have made Pauling's theory untenable to most protein chemists. Recently, the active (Fab) fragments of purified antibody produced by digestion with papain were exposed to guanidine hydrochloride which caused extensive unfolding (Buckley *et al.,* 1963; Noelken and Tanford, 1964). After removal of the denaturing agent a substantial fraction of the initial antibody activity was restored. Later it was found possible to restore activity even after essentially complete reduction of disulfide bonds (Haber, 1964; Whitney and Tanford, 1965). Reoxidation of the disulfide bonds as well as unfolding of the protein was allowed to occur. If, as suggested by physical measurements, the polypeptide chains were completely unfolded during these procedures, the fact that activity was restored represents strong evidence in support of the concept that the final tertiary structure, and hence the antibody specificity, is determined by the primary amino acid sequence.

Further evidence that differences in specificity are related to differences in sequence comes from the work of M. E. Koshland and collaborators who have found small but significant differences among the amino acid compositions of carefully purified rabbit antihapten antibodies of different specificity (Koshland and Englberger, 1963; Koshland *et al.,* 1964). Of particular interest was the finding that a purified antibody directed against a negatively charged hapten contained more amino acids with positively charged side chains than an antibody directed against a positively charged hapten and vice versa. This observation is consistent with the possibility that the observed differences might actually reside in the active sites, especially since other studies have indicated the presence of oppositely charged side chains in the active sites of antibodies directed

against charged haptens. Direct localization of these differences in composition, however, has not yet been accomplished.

Studies with Myeloma and Bence Jones Proteins

One of the most promising avenues of approach to the relationship between specificity and amino acid sequence of antibodies utilizes the products of malignant lymphoid cells. The patient with multiple myeloma characteristically produces large amounts of a protein similar to normal γG- or normal γA-immunoglobulin, but much more homogeneous (Fahey, 1962). Many lines of evidence suggest that isolated myeloma protein is homogeneous with respect to amino acid composition and sequence. In a similar disease, Waldenstrom's macroglobulinemia, large amounts of a homogeneous γM-immunoglobulin (macroglobulin) are synthesized.

As already indicated, the Bence Jones proteins excreted in the urine are homogeneous light chain monomers or dimers derived from myeloma protein or macroglobulin. Homogeneity is of course a prerequisite for meaningful studies of amino acid sequence and attempts to determine the complete sequence of individual Bence Jones proteins are currently in progress in the laboratories of L. C. Craig and F. W. Putnam.

A basic premise of this approach is that the myeloma or Bence Jones protein is a typical, single representative of the heterogeneous γ-globulin population and is not really "abnormal." Presumably, it represents the product of a clone of malignant lymphoid cells derived from a single precursor. In contrast, many clones contribute to the synthesis of normal immunoglobulins of a given class and even to antibody of one specificity derived from a single animal. There is little if any evidence in contradiction to this view and many kinds of experimental data support it. If this theory is correct, studies of amino acid sequence in such "paraproteins" will yield information regarding the extent of variability of sequences among antibodies and should lead to the identification of the variable and invariant regions of the polypeptide chains.

One limitation is that, so far, antibody activity has not been demonstrated in a myeloma protein. This might reflect the difficulty of finding the antigen associated with such a protein. Alternatively, it is conceivable that many or even most γ-globulin molecules are synthesized without any instruction from an antigen and that specificity is an accident of association of a particular pair of light and heavy chains; i.e., many immuno-

globulin molecules do not have a corresponding antigen. In any event, the study of myeloma and Bence Jones proteins may yield, for the first time, precise information as to the degree of heterogeneity of γ-globulins and may result in localization of the active regions of the light and heavy chains.

ACKNOWLEDGMENTS

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Interactions between Plant Viruses and Host Cells

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Introduction

All viruses have two principal features in common, genetic autonomy and the absolute inability to multiply without the help of energy- and metabolite-delivering systems of a host cell. The multiplication of viruses is therefore the result of a chain of close interactions between two genomes, that of the virus and that of the cell.

This chain of interactions is initiated by the infectivity of the virus, which itself must be a function of the intrinsic structure of the viral nucleic acid in connection with particular features of the whole virus particle.

It is evident that the smaller a virus particle is and the simpler its structure, the easier is the elaboration of characteristic features in connection with its function. Many plant viruses are such simple viruses. In addition to having a simple structure they can be easily purified in rather large amounts and exhibit a high degree of variation. Their main disadvantages are failure to show genetic recombination and extremely low plating efficiency. This is why the use of plant viruses for the investigation of problems in molecular biology is restricted mainly to the relation of virus structure to virus infectivity and to some aspects of virus mutation and replication.

The infectivity of virus particles cannot be investigated without the use of an infectible system, and the infectibility of a cell cannot be studied without the infectious virus particle. Although at present some characteristics of virus infectivity and of its genetics may be attributed to certain details of virus structure, the infectibility of the host cell is far from being understood. This is particularly true in the case of the plant cell. The greater part of this review will therefore deal with problems of virus structure in relation to virus infectivity and the genetic determination of

virus strains. Some problems concerning the infectibility of plant cells and the multiplication of viruses will be discussed later.

Structure and Infectivity of Plant Viruses

A. *Minimal Infectious Length of Elongated Plant Virus Particles*

A characteristic feature of many plant viruses (and also of certain bacteriophage found by Marvin and Hoffmann-Berling, 1963, and Zinder *et al.*, 1963) is their overall rod-shaped or threadlike structure. An early observation that the length of most such particles approximates a virus species-specific "most frequent length" or "normal length," immediately raises the question concerning the minimal length necessary for infectivity. For example (Fig. 1), the most frequent length for particles of tobacco mosaic virus (TMV) is about 300 mμ (Williams and Steere, 1951); for wheat streak mosaic virus the length was found to be about 750 mμ (Brakke and Staples, 1958); and for sugar beet yellows virus, about 1250 mμ (Mundry, 1958). The particles of tobacco rattle virus are shorter than those just mentioned (about 180 mμ; Paul and Bode, 1955) and are always accompanied by a second component measuring about 70 mμ. Although the existence of a particular and characteristic length is very striking in all preparations of elongated virus particles, few attempts have been made to correlate particle length with infectivity. This is particularly surprising since for many years the question remained open whether particles deviating in length from the normal length represented common products of virus replication or merely breakdown products resulting from the procedures employed in isolating viruses. In all cases studied, the most frequent length was found—within the limits of estimation—to be identical with the minimal length required for virus infectivity. Investigations on sugar beet yellows virus have yielded evidence that deviation from the minimal length required for infectivity by 100 Å renders the particles noninfectious (Fig. 2) (Mundry, 1958).

According to high resolution electron micrographs obtained by Horne *et al.* (1959) and Russell and Bell (1963), the structure of sugar beet yellows virus particles is fundamentally similar to that of TMV particles. The particles may consist, as in TMV, mainly or even solely of nucleic

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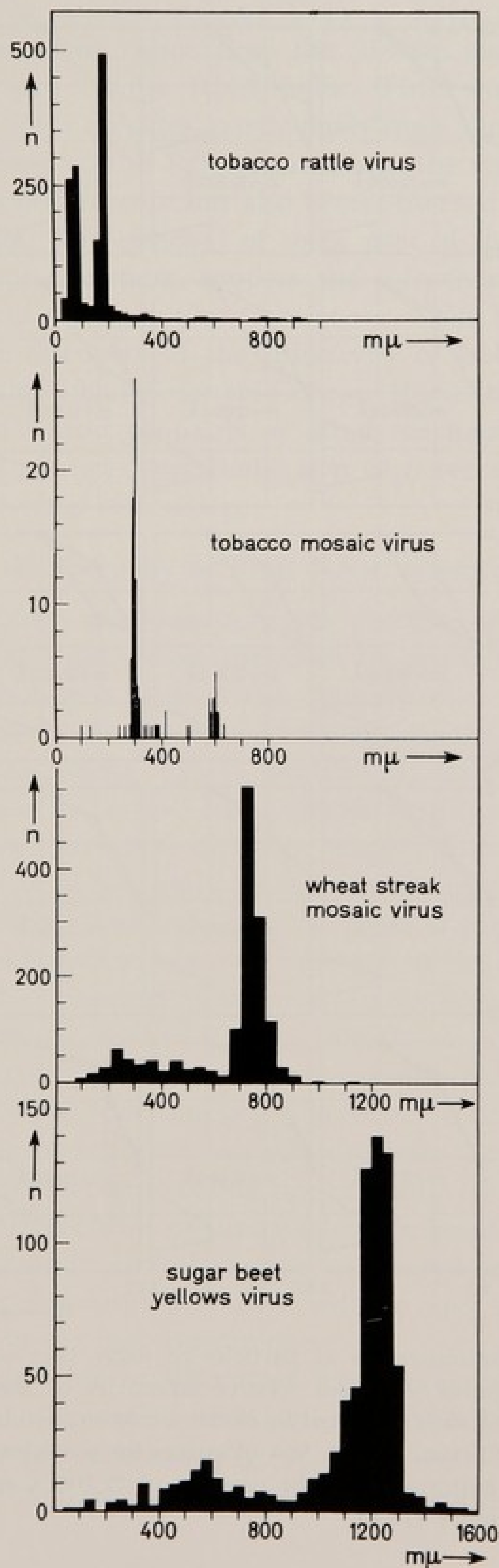
Fig. 1. Frequency of particles of different length in preparations of four elongated plant viruses. Redrawn with data on tobacco rattle virus from Paul and Bode (1955); tobacco mosaic virus from Williams and Steere (1951); wheat streak mosaic virus from Brakke and Staples (1958); and sugar beet yellows virus from Mundry (1958); *n*, number of particles; mμ, length of particle.


500
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250
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tobacco rattle virus
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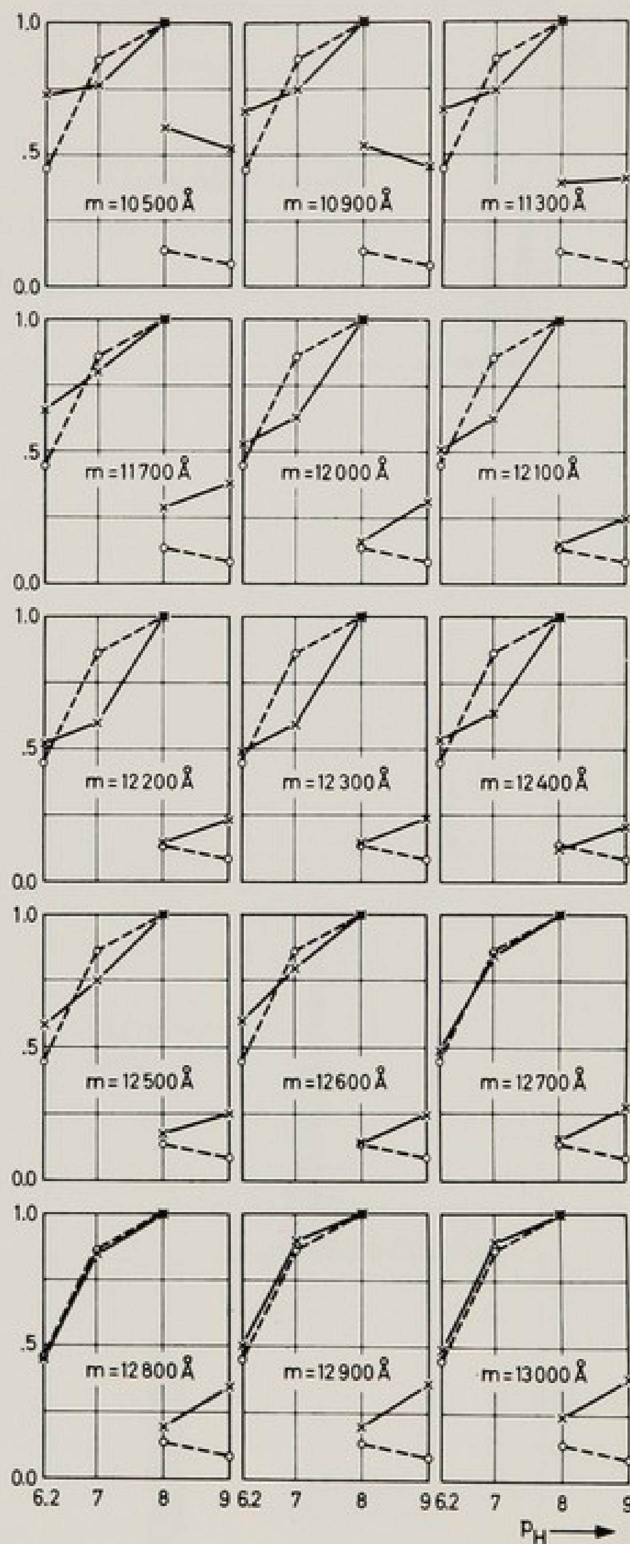

FIG. 2. Relative concentration of particles of sugar beet yellows virus of a given minimal length *m* (solid line) and relative infectivity of the samples (dotted line). Particle concentrations were estimated by electron microscopy via the frequency distribution of particles of different length. Sets of curves for particles of 12,700 Å length and longer superimpose, indicating that the particles of 12,700 Å and longer are the infectious ones. From Mundry (1958).

acid and protein. The nucleic acid of TMV is RNA which is helically twisted and surrounded by subunits of the protein coat; these subunits stack between the turn of the RNA helix. Thus the RNA strand is efficiently protected from all sides, particularly since the protein subunits of the coat extend beyond the RNA strand into the central hollow core of the particle. This hollow core has also been observed in the sugar beet yellows virus particle. The removal of some part of the protein coat of TMV, at the ends or elsewhere, renders the infectivity RNase-sensitive (Hart, 1955; Corbett, 1964; see also Commoner, 1959). This observation was followed by the discovery of the infectivity of isolated TMV RNA (Gierer and Schramm, 1956; Fraenkel-Conrat, 1956). Neither the whole protein coat of TMV (and probably of other mechanically transmitted plant viruses) nor some (e.g., terminal) parts of it seem to play a fundamental role in plant virus infectivity.

B. *The Role of the Integrity of Viral RNA in Infectivity*

The loss of infectivity of elongated virus particles observed on reduction of their original length must therefore be attributed to the loss of some part of the RNA core of the virus. The effect of this loss indicates either the loss of certain genetic information essential for the production of an important component, e.g., a protein intimately related to the replication process, or the loss of some specific terminal structure of the RNA molecule. Such a terminal structure might be related to the initiation or termination of RNA replication without necessarily containing genetic information to be translated into a peptide or protein molecule.

Data from different authors are contradictory in this respect. Today it is well established that cleavage of internucleotide bonds in TMV RNA, either by the action of RNase (Gierer, 1958), or by heat treatment (Ginoza, 1958), always renders the particle noninfectious. Both types of inactivation exhibit first-order kinetics. Therefore, it is concluded that a single break is sufficient to cause loss of infectivity in the RNA strand. Although the results indicate that one out of about 6500 possible breaks is sufficient, the possible effects of internucleotide bond cleavage near an end of the polynucleotide strand cannot be judged from these experiments. The number of nonlethal hits will be too small to cause a detectable shoulder on the inactivation curve.

Fraenkel-Conrat and his collaborators have recently tried to characterize the terminal configuration of infectious TMV RNA by hydrolysis with highly purified exonucleases (Fig. 3). The results are interesting in several respects: (1) Neither end of the polynucleotide chain carries a

phosphate residue (Sugiyama and Fraenkel-Conrat, 1961); (2) the terminal nucleoside at both ends is adenosine in all four strains investigated (Sugiyama and Fraenkel-Conrat, 1963); (3) removal of several nucleotides from the 3′-hydroxyl end of the chain permits certain conclusions to be drawn regarding the nucleotide sequence near this end; also, loss of these nucleotides does not interfere with the infectivity of the RNA strand nor does it influence the primary structure of the virus coat protein subunits. Furthermore, and this observation is most curious, TMV RNA lacking about three nucleotide residues at the 3′-hydroxyl end of the chain and which, according to claims, is still infectious, produces on replication polynucleotide strands of the common structure including the sequence of terminal nucleotides removed from the molecules of the preparation prior to inoculation (Singer and Fraenkel-Conrat, 1963).

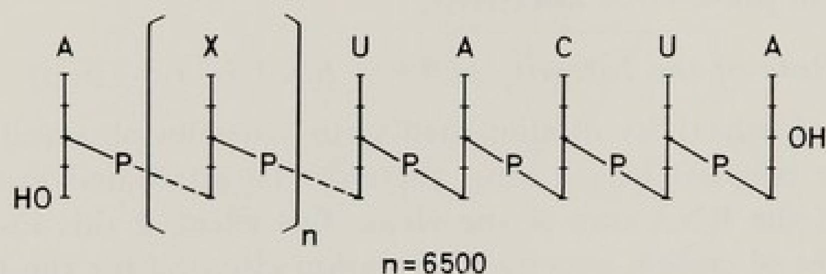

FIG. 3. The terminal nucleotide sequence of the ribonucleic acid of tobacco mosaic virus. Data from Sugiyama and Fraenkel-Conrat (1961, 1963), and Singer and Fraenkel-Conrat (1963).

No sensible explanation is possible at the present time unless it is assumed that the original integrity of the whole nucleotide sequence was preserved in some strands of the preparation and that these strands were the infectious ones that produced the common progeny. It would be worthwhile to study this system again. Only an accurate quantitative and kinetic comparison between the effects of enzymatic attack and the inactivation of infectivity of the RNA, however, could be accepted as proof for the statement that some terminal nucleotides are meaningless in terms of infectivity of the chain. Even then they would not necessarily be meaningless: Their removal might affect a cistron which is nonessential for the infectivity and which then might mutate. A very particular class of mutants should then be found and a wide spectrum of many different types should *not* result from such treatment.

Some calculations have been made concerning the number of nucleotides missing in slightly shortened noninfectious virus particles. For TMV, it has been reported that 50–130 missing nucleotides render the particles noninfectious (Commoner *et al.*, 1958). Density gradient centrifugation of

several spherical viruses often demonstrates the existence of particle classes differing in density. In the case of broad bean mottle virus the most dense particles contain the largest amount of RNA, and only these were found to be infectious. Particles less dense seem to lack 130–360 nucleotides, and are noninfectious (Aronson and Bancroft, 1962).

The very loose structure of the particles of sugar beet yellows virus suggests that single turns of the nucleoprotein particle, and therefore an equivalent amount of its nucleic acid, might be lost upon isolation of the virus. It has already been mentioned that a deviation of 100 Å from the minimal length required for infectivity renders these particles noninfectious (Fig. 2). This was the smallest deviation checked in these experiments, and equals the loss of about four turns of the nucleoprotein particle, according to estimates based on Horne's electron micrographs. Unfortunately, the size of the nucleic acid molecule of this virus has not yet been determined. At the moment we can only speculate that if the RNA is similar in size to many other viruses and has a molecular weight of about 2×10^6 (equaling about 6500 nucleotide residues per chain), the observed inactivation would be due to the removal of about 50 nucleotide residues. Loss of a single turn would then amount to loss of about a dozen nucleotides. These experiments should therefore be repeated with the greatest accuracy possible today.

The structure of polynucleotide chains exhibits a distinct polarity because the phosphate residues form a bridge connecting the 3′-hydroxyl group of one nucleoside with the 5′-hydroxyl group of its neighbor (Fig. 3). A given exonuclease attacks a polynucleotide chain either from its 3′-hydroxyl end or from its 5′-hydroxyl end, according to the specificity of the enzyme. The message contained in a viral RNA strand is read from one end to the other, probably in the same direction in all cases. Removal of terminal nucleotides from the 3′-hydroxyl end, as in Fraenkel-Conrat's experiments, must therefore have an effect fundamentally different from chain curtailment at the 5′-hydroxyl end. In the latter case the first "letters" (so to speak) of the message are lost, and in the former case the last ones are missing. If we are not mistaken in believing that the loss of a few nucleotides in the 3′-hydroxyl terminal sequence does not interfere with the infectivity of viral RNA, then the removal of nucleotides from the 5′-hydroxyl end has a greater probability of being lethal in terms of infectivity.

Practically all the observations (except removal of three 3′-hydroxyl terminal nucleotides) support the view that the entire RNA chain is required for infectivity. Hence the question remains whether missing

information can be complemented by a phenomenon similar to multiplicity reactivation observed with phages.

Very recently, Hulett and Loring (1965) made an interesting observation: According to their data the infectivity of TMV preparations is not dependent solely on the concentration of typical 300 mμ particles. Preparations with a higher concentration of shorter particles than controls with an equal concentration of ordinary particles were more infectious than was expected on the basis of concentration of 300 mμ particles alone. That the effect might be due to a multiplicity phenomenon seems to be a reasonable explanation. There are, however, difficulties in this interpretation. The probability of introducing more than one virus particle into the same epidermal cell during the inoculation procedure is very low since the maximum number of infectible sites per leaf is much smaller than the number of epidermal cells. As will be explained later, the concentration of infectious virus particles in the inoculum must be about 1 mg/ml in order to saturate all infectible sites. To introduce several particles (each smaller than the typical TMV particle) into the same cell, the concentration of such particles must be as high or even higher. The concentration of inoculum used by Hulett and Loring was much lower. Aggregation of particles is therefore required to ensure the introduction of more than one particle at a time into a cell, otherwise this phenomenon remains obscure.

In summary, we can say that the integrity of practically the whole nucleotide sequence of viral RNA is generally needed for its infectivity. The loss of a few percent (or even less than 1%) of the nucleotides from one end interferes with infectivity in the cases studied so far. Perhaps the removal of a very few nucleotides from the 3′-hydroxyl end of the chain may not produce the same results. The possible mutagenicity of this effect has not been checked. The fundamental difference between the two ends of a viral polynucleotide chain as deduced from the polarity of the chain also remains to be demonstrated.

Virus Mutation

Even when the integrity of the whole nucleotide chain of plant viruses is required for the infectivity of these viruses, certain single nucleotides whose replacement by another nucleotide would result in mutation are not necessary for infectivity. Others might not participate in demonstrable genetic information at all. These problems are discussed in the following section.

A. *Virus Mutation in Vivo: Replication Errors and Growth Temperature*

In living cells for which genetic information is mainly conserved in chromosomes, mutations may arise for many different reasons including disturbances of cell division and chromosome partition, and disturbances of chromosome structure and replication. So far, the simple plant viruses have not yielded clear evidence for recombination events (see, however, Aach, 1961; Best, 1961) or multiplicity reactivation, and their infectivity is lost on the occurrence of single breaks in the nucleic acid strand. The mutations of plant viruses which do occur are therefore probably not the result of mere breakage and reunion of RNA fragments. Most if not all mutations of simple plant viruses occurring during virus multiplication must be considered replicating errors, possibly resulting from mispairing of bases and having the effect of base replacements. We might expect that the frequency of mispairing, if it occurs at all, is temperature-dependent. Thus, the mutation rate should be temperature-dependent. In bacteriophage, spontaneous mutation was not affected within a more or less physiological range of temperature (Wittmann, 1957).

We have investigated the effect of temperature on the appearance of variant strains of TMV while the virus was multiplying in tobacco plants (Mundry, 1957b). At temperatures exceeding 30°C a sharp increase was found in the number of tissue areas containing mutants ("mutant spots") produced per gram virus finally isolated (Fig. 4). From 17° to 30°C the number of spots per gram virus was virtually constant. Lower temperatures were not tested. Although selection phenomena are difficult to judge, they do not seem to be responsible, for the following reasons: (1) The types appearing with increased frequency at increased temperatures do not reveal evidence for positive selection; they remain imprisoned in small areas of tissue which are surrounded by tissue infested with the wild-type strain; (2) they do not "overgrow" the wild-type strain even under conditions in which the probability of becoming predominant in the plants is great (that is, when the progeny virus moves out of the inoculated leaves and spreads into noninfected parts of the plant, particularly into the upper noninoculated leaves); (3) short-time exposure of infected plants to a temperature of 35°C for periods of 24 hours produces the most pronounced effect if such treatment is applied immediately after inoculation and before the onset of massive invasion by progeny virus from noninoculated parts of the plant (Fig. 5). This indicates that the very high selection pressure resulting from the rapid multiplication of

the wild-type strain must be maintained at a low level, otherwise, the mutants would not be able to invade enough cells to become detectable with the naked eye.

A reasonable explanation for the discrepancy between these results and the observations on bacteriophages cannot be given. It is possible that errors are more easily produced during replication of RNA than during

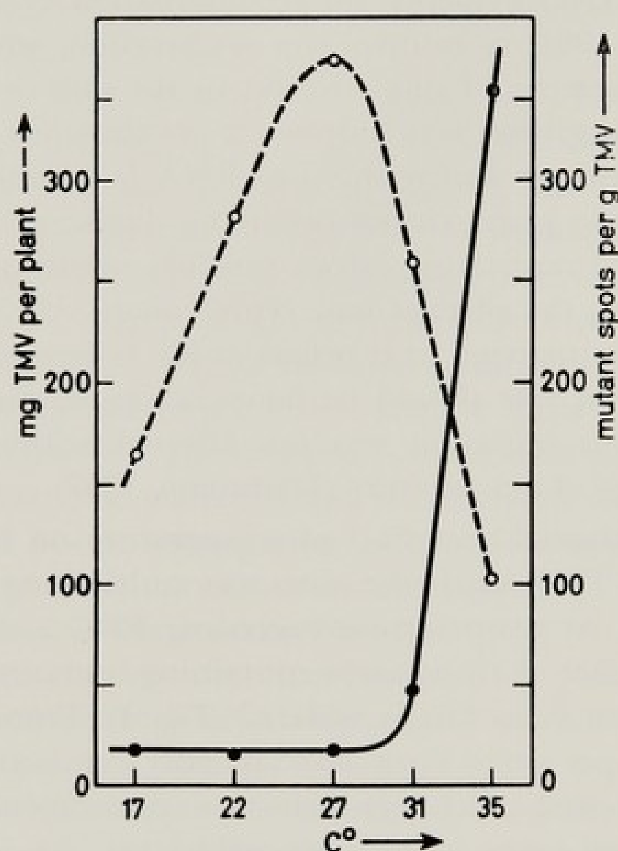

FIG. 4. Mutation of tobacco mosaic virus, strain "vulgare," and the amount of virus produced per plant as a function of temperature. Plants were inoculated and then kept under continuous light at the temperatures indicated. So called "yellow-spots" (small leaf areas infested with mutants, varying in appearance from light green to bright yellow) were counted 15 days after inoculation; dotted line, milligrams TMV produced per plant; solid line, mutant spots per gram virus produced. Redrawn with data from Mundry (1957b).

replication of DNA. The problem concerning the variability in the translation of RNA-contained messages into amino acid sequences (Friedman and Weinstein, 1964), indicated by observations of the temperature dependence of the "meaning" of a given nucleotide triplet, might be considered as evidence for temperature dependency of mispairing among ribonucleotides. Experiments with RNA phage might perhaps clarify the situation.

B. *Conversion of One Nucleotide into Another: Chemomutagenesis with Nitrous Acid*

While irradiation of isolated TMV with ultraviolet light of X rays did not reveal any indication of mutagenic effects (Mundry, 1957a; 1960b), chemical modification of RNA did. Schuster and Schramm (1958) found

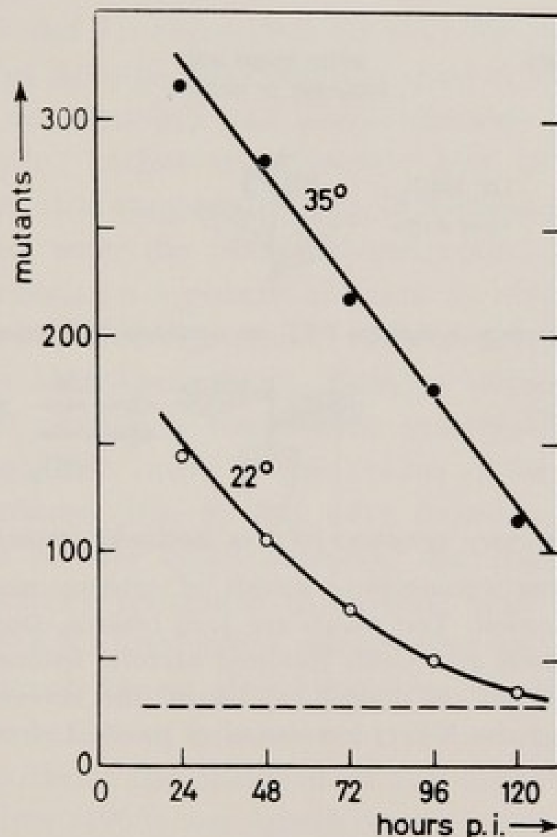

FIG. 5. Influence on the mutation of TMV of the timing of 24-hour exposure to 35°C, of TMV-inoculated tobacco plants. Time 0 = hour of inoculation. Sets of 21 plants were kept in the greenhouse (average temperature 22° ± 2°C) and were exposed to 35°C and continuous light in a climatron for one of the first five days after inoculation. Another series of sets received similar climatron treatment at 22°C. The dotted line represents the average number of "mutant spots" (for explanation see Fig. 4) on two control sets kept in the greenhouse continuously. Data from Mundry (1957b); ordinate, mutant spots per 21 plants; abscissa, hours post-inoculation.

that nitrous acid inactivates TMV RNA without splitting internucleotide bonds. Such treatment results in deamination of the amino bases adenine, guanine, and cytosine, and yields hypoxanthine, xanthine, and uracil, while the integrity of the RNA molecule is maintained. Schuster and Schramm found no pronounced difference in the deamination rates for the three bases when isolated RNA was incubated. Inactivation of

infectivity followed first-order kinetics. Single deaminations thus seem to be efficient in causing noninfectivity of the RNA strand. From the data they calculated that there were 3000–3300 possible lethal hits. This figure is significantly lower than the 4500 deaminations possible per TMV

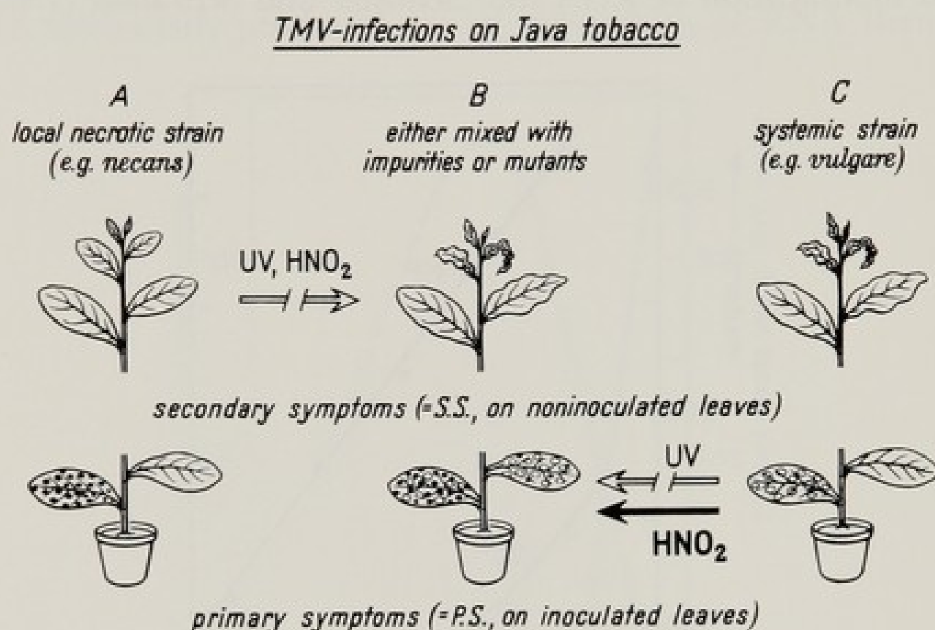

FIG. 6. Mutation from non-necrotic strains of tobacco mosaic virus to necrotic strains, and reverse mutation. Test plants are Java tobacco. Only treatment of a non-necrotic strain with nitrous acid yields localized necrotic lesions among faint chlorotic lesions of the wild type on the inoculated leaves; the reverse mutation cannot be induced; ultraviolet (and also X-ray) irradiation of purified virus is without mutagenic effect in both directions.

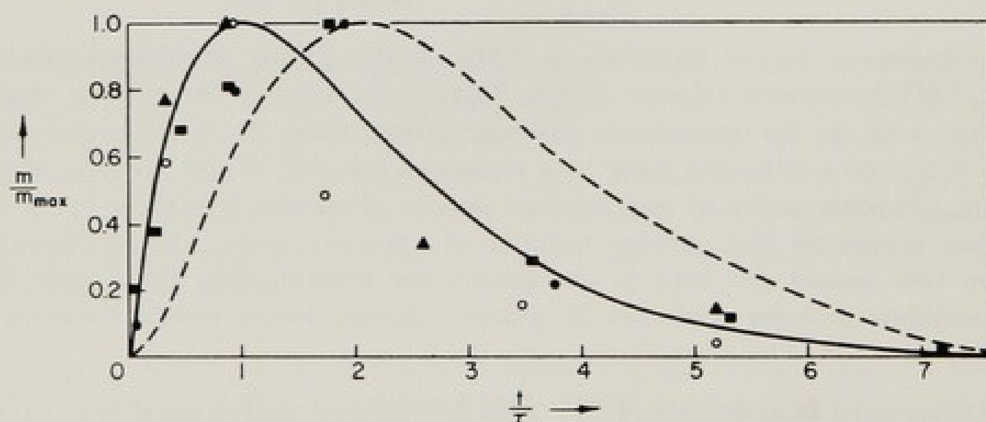

FIG. 7. Dependence of the concentration of mutants (number of necrotic lesions on Java tobacco relative to the maximum value) on the duration of incubation with nitrous acid. t/τ, units of time required for inactivation to e^{-1}, e^{-2}, e^{-3} . . . e^{-7} survivors; under the conditions of these experiments τ is about 18 minutes; ▲, data for TMV nucleoprotein particles, all others for isolated TMV RNA. Data from Gierer and Mundry (1958).

RNA strand. The remaining 1200–1500 amino bases either do not contribute to the genetic information of the virus, or deamination of at least some of them should be mutagenic.

This possibility was investigated and mutants were in fact found (Gierer and Mundry, 1958; Mundry and Gierer, 1958). We checked for the appearance of localized necrotic strains in preparations of RNA from a non-necrotic wild-type strain as a function of duration of treatment with nitrous acid (Figs. 6 and 7). First-order kinetics for inactivation and also for the appearance of necrotic mutants was observed. These results indicate that a single deamination per polynucleotide of 6500 nucleotide residues is mutagenic. Earlier experiments had investigated selection processes and showed that they could be well controlled (Mundry, 1957a).

These experiments were the first demonstration of the mutagenicity of single base alterations on a genetic element *in vitro*. Soon after publication the interpretation of the results was criticized, first by Bawden (1959), and later by Markham (1963). Bawden argued that selection of strains with reduced sensitivity toward the inactivating effect of nitrous acid might have been the cause of the results observed. Such selection processes were calculated (Fig. 8) and were found not to fit the experimental data at all, neither quantitatively, nor qualitatively (Mundry, 1959). Even when *entire* resistance to nitrous acid was ascribed to all (0.4%) mutant type RNA molecules pre-existing in the incubation mixture, 75% of all mutants found after treatment would still have resulted from the mutagenic action of nitrous acid. This argument was rejected by Bawden (1961) without the addition of any other viewpoint.

Markham's criticism (1963) was based on the phenomenon of mutual exclusion among related strains of a plant virus: One strain will not multiply in a cell where a second strain is already multiplying. This leads to the following effects: The lower the concentration of infectious particles is in an inoculum, the further apart are the infections on the leaves, and the lower is the probability that pre-existing mutants or impurities consisting of other strains will be obscured by the bulk of the material. The only reply to arguments involving mutual exclusion is that all our conclusions are based on comparisons of treated samples with controls diluted to give approximately identical spacing of infections on the leaves. Details of this procedure were worked out before the nitrous acid experiments (Mundry, 1957a). They were presented again in connection with a critique of Bawden's argument (Mundry, 1959). Finally, mathematical treatment of the data included an additional correction that expressed and compared the results on the basis of *concentrations* of

infectious particles. Any selection would have caused a deviation from the theoretically calculated curve, but none was observed (see Fig. 7).

Treatment of TMV RNA with nitrous acid yields many different types of mutants. For the appearance of localized necrotic lesions from a non-necrotic wild-type strain, about 180–200 single deaminations were found to be effective. In a screening experiment where as many different mutants as possible were classified, the "total mutability" was found to be several times higher than that towards necrotic strains only. It exceeded the rate

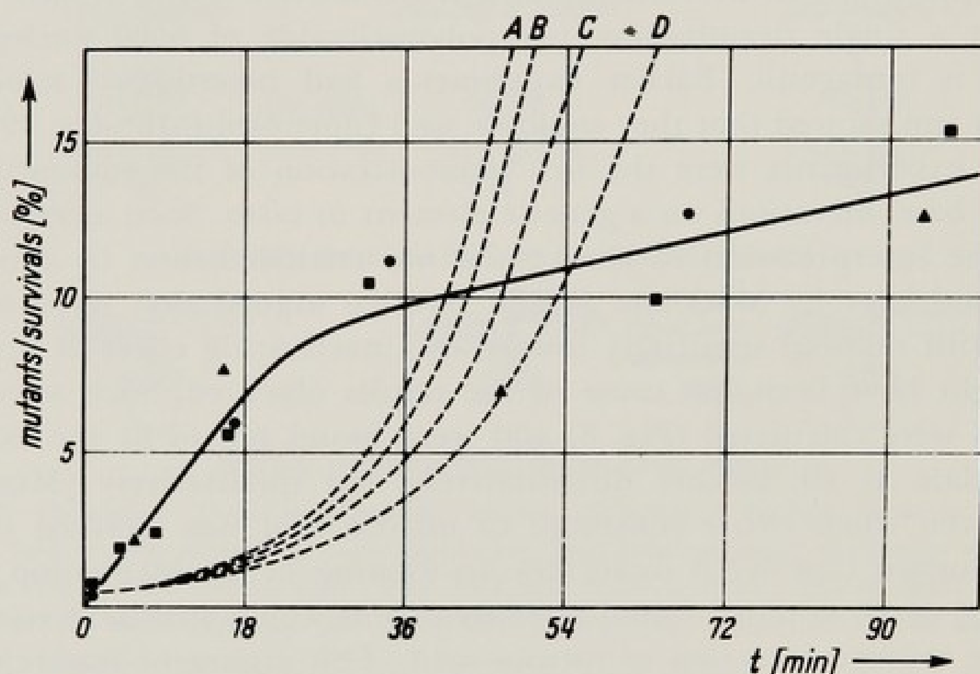

Fig. 8. Production of mutants of TMV RNA by deamination with nitrous acid and theoretical course of selection of more slowly inactivated strains. A,B,C, and D: 0.4% of the infectious material assumed to be entirely resistant (A); twenty times (B); five times (C); and three times (D) less sensitive than the bulk of the material to the inactivating effect of HNO_2; solid line, experimental curve. From Mundry (1959).

toward necrotic mutants by about a factor of 8. To the 3000–3300 "lethal hits" calculated by Schuster and Schramm, we can now add a number of about 1400–1600 mutagenic hits. Hence deaminable nucleotides in TMV RNA not participating in demonstrable genetic information could not be detected. On the basis of several observations (Mundry, 1960c, 1963; Siegel, 1960), we might argue that such nucleotides do not exist. However, according to the degeneracy of the genetic code, nucleotide triplets should exist which would not reveal any biological effect after the deamination of a single base (see Fig. 9). It seems reasonable to assume that the number of such possibilities in TMV RNA is small and has escaped detection.

C. *Other Studies on Chemomutagenesis in Vitro*

Induction of mutation on viral RNA by chemically defined reactions is of particular interest for investigations of the relationship between the structure of a messenger and the structure of a specific protein. For this reason, chemomutagenesis of viral RNA has attracted particular interest and the experiments with nitrous acid were followed by several other attempts to alter the genetic specificity of viral RNA (and DNA) by chemical means.

Schuster and Mundry (1959) carried out some experiments on the possible mutagenic effect of bromination on TMV RNA with inconclusive results (unpublished data). We have also checked the behavior of TMV RNA toward dimethyl sulfate. From the kinetics of the inactivation (first-order kinetics) and the observation that the proportion of mutant types among the survivors remained virtually unchanged, we have concluded that treatment of TMV RNA with dimethyl sulfate is not mutagenic (Table I) (Schuster and Mundry, 1958).

A contrasting conclusion based only on the isolation of variant strains from leaves inoculated with dimethyl sulfate-treated TMV RNA was drawn by Fraenkel-Conrat. Kinetic data were not published, and the possible selection resulting from decreasing mutual exclusion generally leading to an increase in the number of mutant-type lesions has obviously not been taken into account (Fraenkel-Conrat, 1961). For this reason we regard our data as the more conclusive.

The effect of bromination of TMV RNA with bromosuccinimide was followed under similar conditions by Tsugita and Fraenkel-Conrat (1962). It was claimed to be slightly but definitely mutagenic.

The observation that hydroxylamine treatment of bacteriophage evokes mutations (Freese *et al.*, 1961) has been reproduced also with TMV RNA (Schuster and Wittmann, 1963). A slight increase in the proportion of variant strains after treatment of TMV RNA with hydroxylamine has been found among the survivors. The reaction is specific for the alteration of uridylic acid residues under appropriate pH conditions.

The Genetic Code

A. *General Features of the Genetic Code. Support of Current Hypotheses by Studies on TMV Mutants*

We have referred to problems concerning the genetic code several times. I shall not present a detailed discussion, but a short extract will be given of what has been achieved in experiments with plant viruses (particularly

TABLE I

Reaction of TMV RNA with Dimethyl Sulfate[a]

Relative virus concentration (survivors)	Duration of treatment (minutes)	Total infectivity (Local lesions on 15 leaves of xanthi *n.c.* tobacco)	Mutant types (Necrotic lesions on 32 leaves of Java tobacco)	Proportion $\left(\frac{\text{Mutant lesions}}{\text{total infectivity}}\right)$	Average proportion
1	0	6221	19	3.06×10^{-3}	3.44×10^{-3}
0.1	0	1568	6	3.82×10^{-3}	
0.01	0	120	0	—	
0.001	0	21	0	—	
1[b]	0	5982	16	2.68×10^{-3}	3.26×10^{-3}
0.18[b]	3	2220	5	2.26×10^{-3}	
0.01[b]	6	1365	3	2.20×10^{-3}	
0.0034[b]	11	513	3	5.84×10^{-3}	
0.001[b]	16	116	0	—	

[a] From Schuster and Mundry (1958).

[b] Calculated via the infectivity-dilution curve.

with mutants of TMV) on some general features of the genetic code. This seems justified because these studies are also related to some problems of plant virus replication.

The general features of the genetic code could be defined if answers were found to the following questions: (1) How many nucleotide residues are required for coding one amino acid residue (problem of code word size)? (2) How many code words can stand for one particular amino acid (problem of degeneracy)? (3) Does a single nucleotide at its location within the sequence of its neighbors participate in coding for only one or for more than one amino acid residue in the gene product (problem of overlap of code words)? (4) Is the code universal?

The observations of Nirenberg and Matthaei (1961) revealed that in an *in vitro* system prepared from *E. coli* a messenger containing only uridylic acid residues provides sufficient information to code for the incorporation of phenylalanine into peptides synthesized by this system; similarly a chain of cytidylic acid residues codes for proline. We have found that treatment of TMV RNA with nitrous acid is mutagenic. One of the events responsible for this mutagenicity is the alteration of cytidylic acid residues into uridylic acid residues. Therefore, it should eventually be possible to induce the replacement of a proline residue in the coat protein of TMV by a phenylalanine residue. Wittmann (1959, 1962), Tsugita and Fraenkel-Conrat (1961), and Tsugita (1962a,b) have performed experiments of this kind. The proteins of many nitrous acid-induced mutants have been analyzed. A total of 49 amino acid replacements were distributed over 42 mutant lines among the 154 mutant strains checked, while 112 mutants did not exhibit any change in the amino acid composition of the coat protein (Wittmann, 1964). Among the exchanges were the following: proline to serine (observed four times), proline to leucine (observed four times), leucine to phenylalanine (observed once); two cases of the replacement of serine by leucine were also found. Changes in the opposite direction did not occur. This observation has two main consequences: First, it lends substantial support to the claim that cytidylic acid residues do participate in coding for proline in a natural messenger, and uridylic residues for phenylalanine. The second conclusion to be drawn from this observation is at least as important: The TMV RNA and not a replica of it with a reciprocal base composition is itself the messenger.

The results also support the triplet hypothesis, at least partly. Proline can be replaced by phenylalanine either by *two* subsequent exchanges (via serine or leucine) or by a chain of *three* events, to serine first, then via leucine to phenylalanine. In the latter case, provision must be made for

three possibilities of deamination in constructing the code word for proline. Since homologous sequences of cytidylic acid residues are sufficient to code for proline, three such residues would allow the change of the proline code word to a phenylalanine code word via three subsequent deaminations.

It was confusing that the same final result, the substitution of proline by phenylalanine, can be achieved in two ways, either via serine or via leucine alone. The current hypotheses therefore take into account the possibility of degeneracy of the code. Degeneration means that different code words exist for one particular amino acid. The scheme in Fig. 9 combines some of the experimental findings with the triplet hypothesis and the obvious degeneracy of the code for cases of proline-phenylalanine

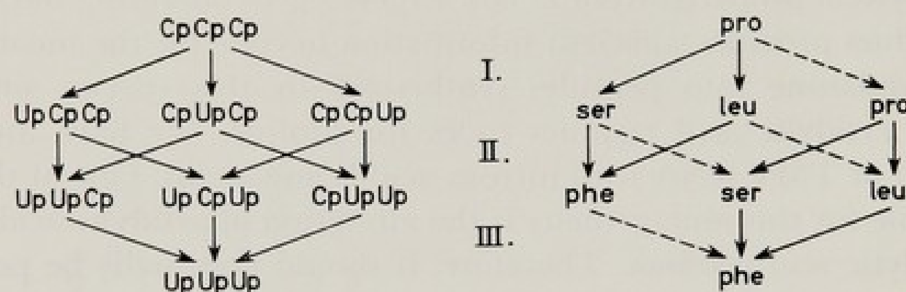

Fig. 9. The deamination of a cytidylic acid triplet to an uridylic acid triplet via three single steps and the change of the meaning of the code words. Dotted arrows represent possibilities for deaminations that do not affect the meaning of a code word and therefore have no biological effect.

substitutions in nitrous acid-induced mutants of TMV. The assignments are paralleled by the results from the *E. coli in vitro* system. Similar suggestions and comparisons could be made for many of the 64 possible nucleotide triplets. Thus, the substitution of serine by leucine can be explained by a change of a triplet UCX to UUX, when X stands for either A or G.

While the triplet hypothesis is reasonable, and the degeneracy of the code well established in the experimental systems so far investigated, the problem of overlap of code words is solved by the data on amino acid replacements in the coat protein of nitrous acid-induced TMV mutants. Simultaneous replacements of two *adjacent* amino acids have never been observed. This indicates that the deamination of a single nucleotide generally causes a change in meaning of only one code word while the neighboring code words remain unaffected. If code words overlap on the polynucleotide strand which represents the messenger the opposite result should have been found. However, among 49 exchanges determined by

Wittmann (1964), not a single exchange of two adjacent amino acid residues could be demonstrated.

Finally it should be mentioned that agreement between the results from two systems so widely unrelated as the TMV-multiplying tobacco cell and the *in vitro* system prepared from *E. coli* cells strongly suggests the possibility that more than the main features of the genetic code is universal. At present there are no reasonable ideas concerning the possible development during evolution of different systems for selection among the code words for a single amino acid. Most of the possible triplets seem to have a meaning in terms of amino acid specification, but it has yet to be shown whether all these are used with equal frequency in all organisms.

B. *The Problem of Functional Separation of Cistrons*

Changes of single nucleotides not affecting the genetic information contained in TMV-RNA can be deduced from details of the triplet code (dotted arrows in Fig. 9), but have escaped experimental detection so far. The question whether there are any should therefore be discussed, and also whether the existence of nucleotide sequences not dealing with the information to be translated into protein is required for the proper functioning of a polycistronic messenger.

TMV RNA, like other viral RNA, is probably a polycistronic messenger. A cistron is defined as a portion of nucleic acid molecule which contains the information for a single protein. A polycistronic messenger hence contains information for several proteins. This information is based on specific sequences of amino acid code words. It is thus clear that the information for different proteins must be kept functionally separated from one another, or the products of adjacent cistra might become interconnected so as to form a super-polypeptide. So far, the general occurrence of a mechanism which splits such a super-polypeptide into its proper polypeptide units, does not seem to be indicated. Cistrons should therefore be functionally separated while they are structurally linked in a polycistronic messenger. These links might be nucleotide sequences carrying "nonsense" in terms of amino acid code words, or they might be links of a non-nucleotide nature.

Several attempts have been made to demonstrate the occurrence in viral (particularly TMV) RNA of non-nucleotide material covalently linked to nucleotides somewhere in the chain, and constituting a part of the backbone of the molecule. No convincing results have been obtained so far. On the contrary, evidence is accumulating that neither amino acids nor metal ions, generally found in traces in even the purest preparations

of viral RNA, constitute genuine components of the RNA backbone. Whether they constitute genuine compounds of TMV or other viral RNA at all, also remains to be demonstrated (see Mundry, 1963).

Recently published data (Brenner *et al.,* 1965) indicate the existence of "nonsense" triplets, to which none of the common amino acids could be related (UAA and UAG). Such sequences would suit very well the purpose of functional separation of cistrons, but their existence in polycistronic messengers can hardly be demonstrated conclusively unless a mechanism has been discovered which permits the selection of triplets according to the natural reading frame.

In spite of this situation, degradation of TMV RNA via a few high-molecular distinct parts, has been repeatedly demonstrated (see Mundry, 1963; Miura *et al.,* 1963). Similarly, the RNA of brome grass mosaic virus seems to occur within the cell as three components (probably not as a result of degradation due to the isolation procedures): The infectious RNA and two pieces resembling in size one-third and two-thirds of the infectious one, characterized by ultracentrifugation analysis (Bockstahler and Kaesberg, 1965). The nature of the weak bonds in viral RNA is still obscure and remains to be investigated, as well as the problem whether the observed distinct pieces of viral RNA are subunits in a functional sense or are mere breakdown products due to intracistronic cleavage.

The Size of Cistrons and the Concept of Minimal Infectious Genetic Information

Based on a triplet code, the size of the cistron which determines the primary structure of the TMV coat protein would be 3 nucleotides for each of 158 amino acids (474 nucleotides).

We have already mentioned that in our nitrous acid-induced TMV mutants, 180–200 deaminations were found, each of which could alter a non-necrotic strain of TMV into a necrotic mutant when the tests were performed on a particular variety of tobacco. The mechanism of lesion production is a hypersensitivity reaction of the host tissue which prevents the spread of virus over large areas and into noninoculated parts of the plant. This mechanism depends on the presence of a single gene in the host cells. It is conceivable that wild-type TMV contains information which counteracts the effect of this host gene, thus enabling the virus to invade the inoculated or otherwise infected plant, and to spread via the vascular system into other parts of the plant. The 180–200 amino bases found to be involved in the induction of necrotic strains by deamination

might very well belong to a particular cistron on the RNA of TMV. This view is supported by the observation that reverse mutations from necrotic strains, including those of spontaneous origin, could not be induced to revert to non-necrotic ones by treatment with nitrous acid (Mundry, 1960a). If allowance is made for the (nondeaminable) uridylic acid residues, the size of this cistron could very well be 250–300 nucleotides.

Investigations of the multiplication of several RNA viruses, including plant viruses (Ralph *et al.*, 1965), has revealed that a specific enzyme not present in the uninfected cells is required to replicate the RNA via a double-stranded RNA. It is assumed that this enzyme is virus-specific, and that information necessary for its production is a part of the viral genome. The smallest RNA virus should therefore possess a cistron for the production of this enzyme and one for the virus' coat protein. Thus an RNA equivalent to at least two cistrons would be required to constitute infectious viral information.

The RNA of the smallest plant viruses so far isolated is much larger than would be anticipated from the above argument. The RNA of broad bean mottle virus contains about 3400 nucleotides (Yamazaki *et al.*, 1961), and the RNA of brome grass mosaic virus has about 3000 nucleotides (Bockstahler and Kaesberg, 1961). The smallest viral RNA is that of a small bacteriophage in which the nucleic acid molecule has only 1600 nucleotides (Loeb and Zinder, 1961, quoted in Caspar and Klug, 1962). This figure is probably close to the lower limit of that necessary to constitute infectious genetic information.

There is, however, another RNA virus with only 1160 nucleotides, apparently below this limit. It will not multiply in healthy cells of its natural host, but it will reproduce if another virus is present in the cells. Multiplication of the tobacco necrosis satellite virus (Kassanis, 1960; Reichmann *et al.*, 1962; Reichmann, 1964) seems to be dependent on the presence of additional information, carried neither by itself nor by the genome of the host cell. It is reasonable to assume that the information missing in the RNA of the satellite virus is the cistron for the RNA replicating enzyme, and that the larger tobacco necrosis virus provides this information upon infection. We can speculate that the minimum number of nucleotides required to constitute fully infectious genetic information is of the order of a thousand.

It should be mentioned that the two species of particles generally found in preparations of tobacco rattle virus (Fig. 1), of which only the larger is infectious (Harrison and Nixon, 1959), are also of interest in connection with the concept of incomplete infectious genetic information.

Plant Virus Replication and Some of Its Cellular Events

In the preceding sections we have tried to define some of the features characteristic of the construction of infectious genetic information, by describing the events which cause the loss of infectivity and change of the original genetic information of a virus. We cannot, however, answer the question why a certain sequence of nucleotides, like the one in TMV RNA, is infectious. It is still unknown how host cells, for example those of tobacco leaves which contain about 2×10^9 base pairs of DNA per nucleus, are forced to reproduce a virus simply by the addition of an RNA strand of only 6500 nucleotides to this mass of information. Observations on the fundamental processes of reproduction of viruses in the plant cell are few and scattered, and are far from fitting into a reasonably established picture. Only a few details of more general interest will be presented here.

The mechanism of virus uptake by plant cells is still obscure. While pinocytosis in animal cells is gaining particular interest in this respect (Smith, 1963), observations on pinocytosis-like phenomena in plant cells are few and lack details. If pinocytosis is the mechanism for the uptake of virus, the saturation of all infectible sites on a plant leaf should take place when the virus concentration in the inoculum is enough for there to be one infectious particle for each microdrop of inoculum within a single pinocytosis vesicle. I have calculated the size of a drop that would contain one infectious particle at a concentration of virus in the inoculum which would assure a maximum number of lesions on the leaves. A droplet size of about 1 μ^3 was found for inoculations with the nucleoprotein particles or independently with TMV RNA (Mundry, 1963). This calculation agrees with the hypothesis of virus uptake into plant cells by pinocytosis.

Although the uptake of viruses by plant cells probably depends upon their physiological activity (particularly when pinocytosis may be involved), it also seems to be dependent upon the nature of the virus coat protein. The specific infectivity of mixed reconstitutions of TMV (that is, TMV rebuilt from isolated RNA of one strain and isolated coat protein of another) parallels the specific infectivity of the protein donor strain, and not that of the strain from which the RNA has been isolated (Holoubek, 1962). The coat protein might therefore have something to do with stimulating virus uptake. It would be interesting to know whether it also stimulates pinocytosis.

Earlier we mentioned the phenomenon of strain interference by mutual

exclusion. Nothing is known about the time of onset of mutual exclusion. If it is based on competition among strains for adsorption sites or entry points for virus uptake into the cell it should occur early in the infection process. This possibility was tested by experiments based on the observation that rubbing leaves with buffer produces wounds or exposes to the environment attachment sites which remain susceptible to infection for a while. These sites can be brought into contact with virus by dipping the prerubbed leaves into the inoculum. In our experiments such sites were subsequently exposed to two different strains. One was a non-necrotic strain at a high concentration which had previously been shown to guarantee saturation of practically all previously produced infectible sites. The other was a local lesion-producing strain, at a concentration which would give proper lesion counts. No difference was found between the results depending upon whether prerubbed leaves were first dipped in strain A and then into strain B containing inoculum or vice versa. The lesions deriving from this treatment were always identical in number to those appearing on the controls in which the dip into the non-necrotic, highly concentrated inoculum was replaced by a dip into buffer containing no virus. If time was allowed to elapse between the first virus dip and the second one no other effect could be observed than a general decrease in the tendency of the leaves to produce infections through preformed wounds. No indication of mutual exclusion was found within 10 minutes after the first dip. Within this time the leaves lost their infectibility. Among the possible explanations of these findings are the following hypotheses: (1) There is no strain-specific adsorption of virus to receptive sites; a certain number of sites are produced, lose their infectibility, and virus adsorbed to them may be replaced by the virus applied during the second dip; (2) there are absolute strain-specific sites, and therefore no competition for infectible sites; again, a number of sites are produced upon rubbing and these lose infectibility with time; (3) the leaf is stimulated by the rubbing procedure and the stimulus leads to the continuous production of very short-lived sites which are nonspecific toward strains; as the stimulus dies, fewer and fewer sites appear, and after 10 minutes, no more. In this case, the sites present during the time of the first dip are not identical with those present at the time of the second dip. These hypotheses could be tested but have not been so far (Mundry, 1958b).

Little is known and little can be said about the fate of the virus particle after it has entered the plant cell. From the following observations we can deduce that the coat protein is removed from the nucleic acid mole-

cule of the virus particle. There is a change in the sensitivity of infective centers on inoculated leaves to the inactivating effect of ultraviolet light (Siegel and Wildman, 1960; Mundry, 1963). Infective centers become sensitive to the action of pancreatic ribonuclease from soon after the infection up to two hours *post infectionem* (*p.i.*) (Hamers-Casterman and Jeener, 1957). In addition, photoreactivation of potato virus X is only possible within 30–120 minutes *p.i.* (nucleoprotein particles are probably not reactivated by light) (Bawden and Kleczkowski, 1955). Hence the protein may have been removed within the first half hour *p.i.* This time may not be the same for different plant viruses. Details of the stripping procedure have been recently reviewed (Mundry, 1963).

Recent experiments on the detection of double-stranded RNA as the replicating form of viral RNA in bacteria have lead to similar efforts in the case of virus infected plant cells (Burdon *et al.,* 1964; Shipp and Haselkorn, 1964; Ralph *et al.,* 1965). The characterization of an enzyme involved in the production of double-stranded viral RNA during the course of infection has been attempted (Weissmann *et al.,* 1964). Although details of the replication mechanism are still unknown it is remarkable that an RNA-dependent RNA replicating enzyme is not ordinarily present in normal cells, but is produced upon infection following the introduction of genetic information with the viral RNA. This hypothesis is supported by elegant experiments on a RNA phage (Spiegelman *et al.,* 1965). It provides a key to the problem of virus infectivity.

Finally some data related to the biochemical events of virus multiplication in plant cells will be discussed. Direct observation of metabolic changes within the infected cell was performed by ultraviolet microspectrophotometry (Zech and Voigt-Koehne, 1956; Zech, 1963, as cited in Mundry, 1963). Zech and his collaborators studied the change in the optical density at 265 and 280 mμ at different locations in the cell after the introduction of TMV. The measurements cover the first 24-hour period after the inoculation. The studies were performed on the cells of tobacco leaf hairs. The terminal cell was inoculated and the changes in RNA concentration or nucleolar mass determined in the cell adjacent to the inoculated one. Careful control and standardization measurements have been published separately (Zech, 1961). The data are summarized in Fig. 10.

About 30 minutes *p.i.* the RNA concentration in the nucleus begins to rise. At about 2 hours *p.i.* it has increased up to 2.3 times its original value. This concentration remains constant until 5 hours *p.i.* then it starts decreasing gradually until 9 hours *p.i.,* when it reaches its original

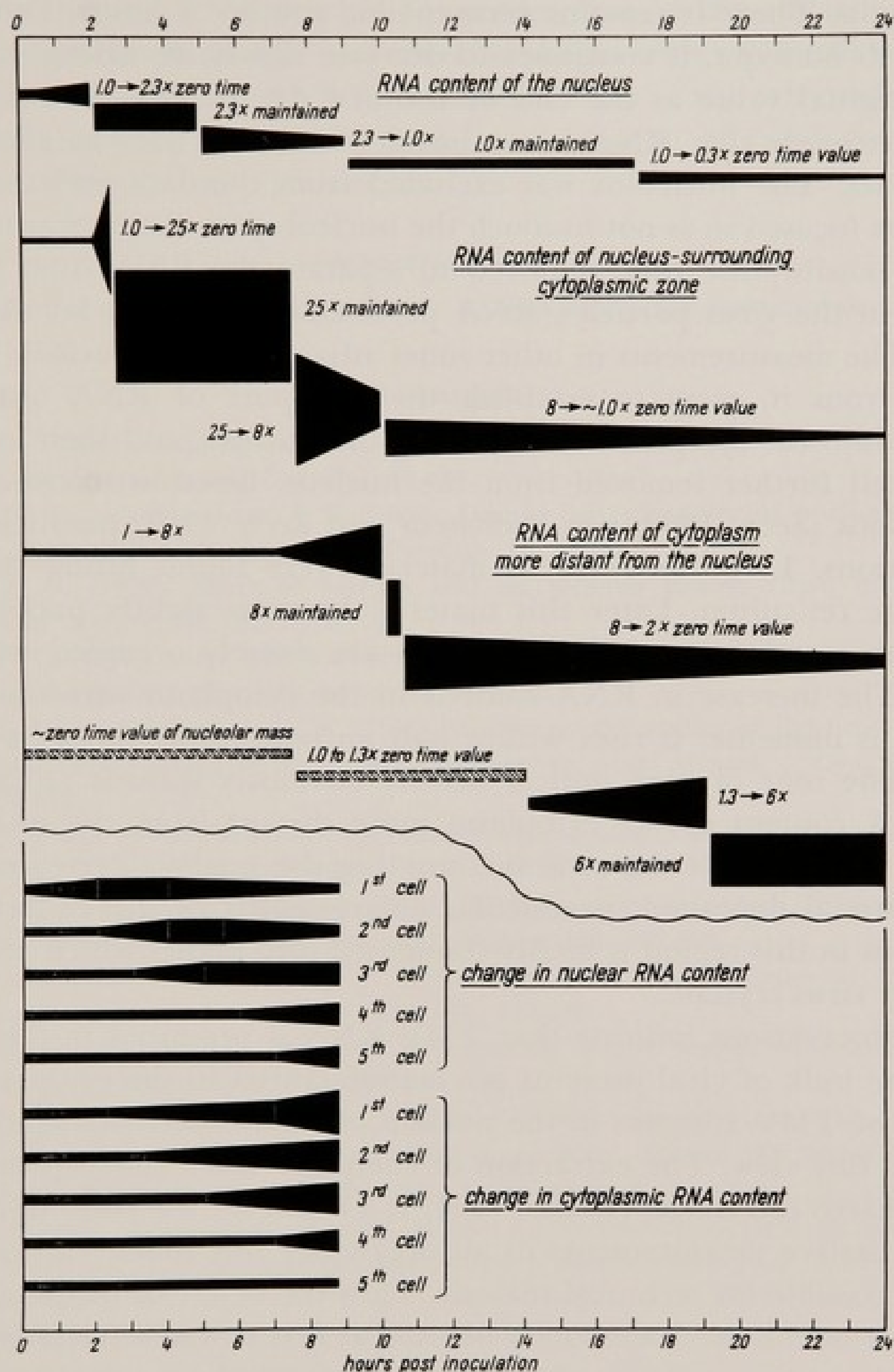

FIG. 10. Ultraviolet-microspectrophotometric measurements on TMV-infected tobacco leaf hair cells. Data from Zech (1963); reproduced from Mundry (1963). The terminal cell of a leaf hair was inoculated at time 0, and changes in RNA content or nucleolus mass in the cell adjacent to the inoculated one were followed. The widths of the bars in each sequence are proportional to the data obtained; each bar represents an average of the data obtained from many (occasionally more than 100) cells; data represented by hatched bars are less reliable due to technical difficulties; below the wavy line numbering begins with the cell adjacent to the inoculated one and proceeds toward the base of the hair.

value again. There it remains constant for another 8 hours. During the eighteenth hour *p.i.* it continues to decrease slowly, revealing only 30% of its original value at the end of the first day of infection. The data suggest strongly that RNA is produced within the nucleus after about 30 minutes. The nucleolus was excluded from the data and the microbeam was focused so as not to touch the nucleolus for these measurements. This is possibly the time required to separate the RNA from the coat protein of the virus particles. RNA production continues for about 4–5 hours. The measurements in other zones adjacent to the nucleus, or more distant from it, seem to establish the transport of RNA out of the nucleus into the cytoplasm surrounding the nucleus, and then into parts of the cell further removed from the nucleus. Electron microscopic investigations (Zech, 1960; von Wettstein and Zech, 1962) paralleled these observations. Large amounts of material were found filling the endoplasmatic reticulum. Later this material becomes tightly packed there, while the membranes of the reticulum are densely occupied with ribosomes. The increase in RNA content in the cytoplasm surrounding the nucleus is immense; it rises within half an hour to 25 times its original value. The zone of high optical density obviously spreads radially, and the RNA content of the cytoplasm more distant from the nucleus increases, while that of the zone surrounding the nucleus decreases slowly. The material deposited in vesicular enlargements of the endoplasmatic reticulum in this region is finally condensed into bodies which are almost certainly virus crystals.

The observations indicate that TMV RNA is produced in the nucleus while the bulk of viral proteins are manufactured in the cytoplasm. The absence of TMV antigens in the nucleus of infected cells (Nagaraj, 1965) supports this view. The extraction of complete TMV nucleoprotein rods from tobacco cell nuclei (Reddi, 1964) is a claim for the contrary.

Quantitative measurements in all organelles and zones within the cell were impossible for technical reasons. Thus the behavior of the nucleolus could not be followed accurately because at first it was surrounded by the highly absorbing nucleus, and later by the very dense plasma zone surrounding the nucleus. Only after the RNA was widely distributed, and the optical density in the cell sufficiently decreased, was it possible to establish that there was a strong increase in the optical density of the nucleolus. Because of the very low water content of the nucleolus, a 30–50% rise in its extinction value could indicate a much higher RNA accumulation or production than would a 200–300% increase in the relatively water-rich, non-nucleolar portion of the nucleus. In spite of these difficulties, the conclusion seems justified that the onset of (non-

nucleolar) nuclear RNA synthesis precedes that of the nucleolus. Viral RNA synthesis in plant cells may therefore begin with some still unknown interactions between this RNA and the chromatin in the cell nucleus. The nature of this interaction is obscure and may be of fundamental importance for regulation phenomena.

Acknowledgment

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Interaction of Viruses with the Genetic Material of the Host Cells

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Introduction

The study of virus biology contributes in two ways to the problem of reproduction and function of genetic material. The first contribution derives from the use of viruses as model systems for investigating the reproduction and functionality of nucleic acids. Viruses have various types of nucleic acids, some of which, such as single-stranded DNA and RNA or double-stranded RNA, are either absent or not self-reproducing in cells; their reproduction can be analyzed in great detail. The second contribution derives from the consequences effected by viruses on the multiplication and function of genetic material in the cells they infect.

This presentation will be mainly concerned with the latter aspect of virus biology; it cannot, however, be totally dissociated from the former because the consequences for the cells deriving from virus infection depend on the special nature of viruses. Viral replication within a cell is not an independent and isolated event; it is rather the result of a profound rearrangement in which both the virus and the cell lose their independence and are incorporated into a new biological entity, the virus-cell complex. A new set of enzymes and regulatory proteins specified by viral genes are formed in the cells; in many cases, they bring fundamental cellular functions to a standstill and initiate new virus-controlled ones. For instance, the synthesis of cellular DNA or cellular messenger RNA may be halted and replaced by the synthesis of viral nucleic acids; or, the cellular DNA may be destroyed. In cases in which the cells are not as drastically affected, the regulatory pattern of macromolecular synthesis is often greatly altered. Biosynthetic or energy-yielding pathways continue to operate, but become utilized in whole or in part for the synthesis of viral materials.

Virus-cell complexes differ from uninfected cells in anatomical properties also: Special membranous structures are recognizable, for instance, in cells infected by polio virus. There is abundant evidence for the occurrence of important structural changes in the cellular membrane caused by incorporation of virus-specified proteins. Finally, the anatomy of the genetic material itself, the DNA of the cellular chromosomes, can be changed, for example, by the insertion of the viral DNA.

The infected cells are in most cases noticeably different from the noninfected ones; this phenomenon is defined as virus-induced *conversion*. If the cells are ultimately killed by the virus, conversion becomes detectable in the period between infection and cell death; if they are not killed, conversion may be continuously present. Many symptoms of conversion are known: changes of surface antigens, production of special products such as toxins, modification of the permeability of the cellular membrane, and loss of sensitivity to factors regulating cell multiplication. The latter phenomenon, which is defined as "transformation," is especially evident in animal cells infected by cancer-producing viruses.

We can now turn more specifically to the subject of our discussion, the consequences of virus-cell interaction on the cellular genetic material. There are three main classes of consequences to which we shall devote our attention: (1) anatomical, resulting from insertion of viral DNA in the cellular DNA; (2) functional, resulting from interference in the operation of cellular genes; (3) degradative, resulting from breakage or breakdown of the cellular DNA.

Consequences of Virus Infection for the Genetic Material of Bacterial Cells

These consequences will be considered first since they have been extensively studied and offer a suitable model for analyzing the much less complete results obtained with animal cells.

Anatomical Consequences

The insertion of the viral DNA in the DNA of the host cells has been extensively studied in *Escherichia coli* cells infected by the temperate bacteriophage λ. The molecular events involved in the insertion are known in considerable detail; they will now be presented in a rather idealized fashion which includes a certain amount of speculation.

The DNA of bacteriophage λ enters a previously uninfected cell in the form of a linear molecule comprising about 1000 nucleotide pairs. After

entering the cell it assumes the shape of a ring, first by pairing of the two ends which possess complementary single-stranded regions (Hershey *et al.*, 1963), and then by establishing covalent bonds. The ring is the reproductive form of the virus. Duplication of the molecule probably occurs after transient opening of one of the links which close the ring; then the molecule has a swivel point with free rotation permitting the disentanglement of the newly produced molecule from the pre-existing molecule. These molecules are also undergoing recombination in which parts deriving from two different molecules unite to form a new molecule. Recombination can be analyzed in classic genetic terms to determine the order and distance of genes of a viral genome. This analysis permits the establishment of a linear genetic map which is called the vegetative map since it is obtained from the autonomously replicating or vegetative form of the viral DNA; the ends of the map coincide with the ends of the linear molecule as it exists in the extracellular virus particles.

The vegetative λ DNA can become inserted into the bacterial chromosome by a process known as lysogenization. Insertion occurs at a special site on the bacterial chromosome and is believed to occur through the recombination of two homologous segments of viral cellular DNA after the viral and cellular DNA have been broken at determined points by a virus-specified nuclease of very high specificity. The inserted viral DNA is now called a prophage. The order of the prophage genes can be determined by genetic means to yield a prophage genetic map. This map is linear also, but differs from the vegetative map in that a block of genes present at one end of the molecule in the vegetative map is shifted to the other end in the prophage map (Calef and Licciardello, 1960; Campbell, 1963; Franklin *et al.*, 1965) indicating that the point at which the viral DNA ring opens for integration is different from the one at which it opens during vegetative multiplication.

The prophage is an intrinsically unstable system because it contains a mechanism for its own termination in the form of a gene that specifies the nuclease. The nuclease that is instrumental in causing the breaks leading to insertion can cause similar breaks in the DNA of the lysogenic cells; by recombination at these points, the prophage can be freed from the bacterial DNA in the form of a vegetative ring; this process is the exact reverse of the one causing insertion. The nuclease, however, is not normally made by the prophage gene, owing to the operation of the immunity gene of the prophage itself (Jacob and Monod, 1961). The immunity gene causes the synthesis of an immunity repressor which prevents the expression of many prophage genes, including the one spe-

cifying the nuclease (Korn and Weissbach, 1964). The concentration of the repressor is maintained by a steady state in which loss by thermal inactivation and cell multiplication is compensated by new synthesis. Under normal conditions, the system is well balanced; if it is thrown off balance, the nuclease gene is activated and, by a process called induction, the prophage is converted to the vegetative form.

Induction not only causes the formation of a free and complete vegetative viral DNA, but can also yield a complete cellular chromosome free of the prophage. This restitution is possible through the recombination events already discussed.

The insertion by recombination and the recovery of the inserted DNA, as well as the cellular DNA, in complete form, can occur only because the viral DNA has a circular shape; the insertion of a linear molecule would require two recombination events and the loss of the intervening segment of cellular DNA. Furthermore, since the recombination events presumably could not take place at the ends of the viral molecule, insertion would result in the loss of two terminal segments if the viral DNA were linear. Thus, circularity appears to be an essential condition for integration; for this reason, any circular viral DNA is suspected of insertion in the cellular DNA.

The prophage can undergo a different type of recombination event with the host DNA adjacent to the insertion site. Two nonreciprocal crossovers are involved, in which the amounts of DNA exchanged are not equal. The consequence of this event is the formation of a new type of prophage called λdg which incorporates a segment of cellular DNA, but has lost a part of its own complement. The cellular segment carries the genes of the bacterial galactose operon, and the viral segment lost contains several genes. For this reason, the λdg molecules are defective, i.e., they cannot initiate the formation of virus by themselves, but can do so in cells infected at the same time by normal λ which supplies the functions of genes missing in the λdg molecules. The λdg molecules are physically different from normal λ molecules.

Replication of the prophage is under the control of mechanisms that carry out replication of cellular DNA; the prophage is replicated every time the synthesis of the bacterial DNA, which proceeds from one end of the chromosome to the other, reaches the site of the prophage (Nagata, 1963). An important consequence is that mutated viral DNA, unable to multiply vegetatively, can multiply in the form of a prophage (Jacob *et al.*, 1957).

The finding that viral DNA can become inserted in cellular DNA is of

extreme interest, since it is relevant to the question of the evolution of viruses and of the meaning of virus-host relationships. In order to assess its significance, the problem of mechanics must be considered: How is the attachment and insertion of a viral DNA molecule at a specific site of the cellular DNA possible? This question is answered by experiments indicating that the DNA of bacteriophage λ and that of *Escherichia coli* cells contain areas of homology; i.e., areas in which the two DNA's have either identical or very similar base sequences; about 34% of the viral DNA and 0.2% of the cellular DNA are involved (Cowie and McCarthy, 1963; Green, 1963). Homology exists, not in a single segment of the viral DNA, but in several segments present in different parts of the molecule (Cowie and Hershey, 1965). The presence of these homologous segments explains how the viral DNA can attach at a specific site on the cellular DNA; at the same time, however, it raises the question of their origin. The extensive homology which involves several parts of the viral DNA molecule shows that a segment of the cellular DNA, approximately equivalent in length to a λ DNA molecule, is greatly similar in base sequence to λ DNA, but not identical. It is likely that at a certain time in evolution the viral DNA and the corresponding segment of the cellular DNA were identical and became progressively differentiated later on, retaining, nevertheless, considerable similarity. Thus, either the virus DNA derives from a segment of the bacterial chromosome or vice versa; the λ-specific segment of the *Escherichia coli* DNA derives from the irreversible insertion of λ DNA.

The second possibility appears much more likely. Defective λ DNA can be irreversibly incorporated; if the molecule has lost the part containing the nuclease and the immunity genes it cannot be released by induction and is functionally silent; it is therefore unrecognizable, except for the possibility of rescuing some of its remaining genes by recombination with a properly marked λ (Fisher-Fantuzzi and Calef, 1964). If the association has existed for a long time and many mutations have taken place in the inserted DNA, even gene rescue may become impossible. Insertion of the DNA of episomes (elements related to viruses) in the bacterial DNA at many places, possibly without specific sites, is known to occur, but there is little probability that it will; after the insertion has taken place once, however, an attachment site for the episome is created, and attachment to it of the same episome occurs with much greater probability (Campbell, 1962).

What is the significance of lysogeny? The complex genetic system that makes it possible appears to have had a long period of evolution and

therefore appears to be selectively advantageous for the virus; its selective value, however, is not obvious in terms of survival since a lysogenic bacterium is not more resistant to the environment than a free virus particle. The selective value may be more subtle and derive from the fact that the lysogenic state favors virus reproduction by conserving the host cells, in contrast to lytic viral multiplication which kills the host cells.

Functional Consequences

The insertion of a prophage in the bacterial DNA affects the state of regulation of neighboring bacterial genes. Lambda prophage, for instance, affects the control of the genes of the galactose operon which is adjacent to the prophage (Buttin *et al.*, 1960; Yarmolinsky and Wiesmeyer, 1960; Buttin, 1963). The genes of the operon are normally under the control of a repressor substance produced by a bacterial regulator gene which acts on the operator gene and prevents the expression of the genes of the operon; the action of the repressor is counteracted by proper inducing substances. In cells lysogenic for λ, the degree of repression of the galactose operon is increased and less enzyme is produced, as if the system has become sensitive to a new repressor, for instance, the immunity repressor of the prophage itself. When the lysogenic cells are induced and the effect of the viral immunity repressor decreases, the repression of the galactose operon is also decreased; a burst of enzyme synthesis occurs although the normal bacterial repressor specific for the galactose operon is present in the cells. These and other phenomena suggest that the bacterial operon falls under the control of the regulation system of the prophage.

Another important consequence of the insertion of a prophage for neighboring bacterial genes is a mutagenic effect. This has been demonstrated for the bacterial virus Mu 1 (Taylor, 1963) which lysogenizes *Escherichia coli* cells. Nonlysogenic cells infected with this phage undergo a short period of intense mutagenesis following infection at the time insertion of prophages occurs. The mutagenic action affects a variety of genes; in each case the prophage appears to be inserted near the mutagenized gene. This virus is thus different from λ in that it does not have just one attachment site; either it has many sites, or no site at all and becomes inserted at random. Whether mutation is caused by a functional, potentially reversible effect similar to that of prophage λ on the galactose operon or by a permanent structural modification of the bacterial genes is not known.

Degradative Consequences

Breakdown of the host cell DNA is caused by some bacterial viruses which do not cause lysogenization; the breakdown is complete and yields small molecular products which are then utilized for synthesis of the viral DNA. The mechanism is unknown.

Consequences of Virus Infection for the Genetic Material of Animal Cells

The amount of factual information concerning these consequences is much less than that available for bacterial viruses. Thus, interpretation of the findings is much more speculative and is based on inferences drawn from the knowledge of the bacterial virus systems. In order to do this, it is perhaps useful to dispel the common misconception that bacterial viruses are profoundly different in properties from viruses of higher organisms, especially in their ability to give rise to lysogenic complexes. Striking observations deriving from genetic studies of animals and plants, however, very strongly recall the functional consequences of prophages. The observations concern the regulator elements of corn (McClintock, 1961) and heterochromatin. These elements are transposable from one chromosome to another by regular chromosomal mechanisms such as translocation and perhaps by independent migration also; their effect on the neighboring genes is similar to that of prophage. Three consequences are known: reversible inactivation of gene function, mutagenesis, and breaks. The first two consequences find a counterpart in the functional consequences of prophage; the last mentioned may be analogous to the breaks that lead to the formation of the defective transducing virus particles such as λdg.

Two consequences of viruses for the genetic material of animal cells will be discussed now. One is the production of chromatid breaks caused by many viruses; the other is the production of hereditary transformation by tumor-producing viruses.

Chromatid Breaks

Although many animal viruses induce the formation of chromatid breaks in animal cells (Fjelde and Holtermann, 1962; Hampar and Ellison, 1963; Stich *et al.*, 1964), very little is known about the mechanisms by which these breaks are produced. In some cases, the breaks are formed before the cells are killed and may be the expression of early

autolytic phenomena. More interesting are the breaks induced in multiplying cells, as in the permanent MCH line of Chinese hamster cells infected by herpes virus (Hampar and Ellison, 1963). These breaks are formed at characteristic locations in the chromosomes (Stich *et al.*, 1964); the location of the breaks is similar to that of breaks induced by 5-bromodeoxyuridine, but different from the location of those induced by hydroxylamine or X rays. These regularities suggest that the site of the breaks is dependent in part on the properties of certain chromosomal sites rather than deriving from an insertion of the viral DNA. It is likely that the location of the break sites depends mainly on two factors: the local chemical composition of the DNA and of its environment; and, the state of regulation of the DNA segment in respect to either function or replication. Further elaboration of the hypotheses seems to be unwarranted.

Chromatid breaks, possibly of different significance, are formed in secondary cultures of hamster embryo cells during the early stages of transformation by polyoma virus (Vogt and Dulbecco, 1963). Again, the mechanism of production is unknown. The interesting aspect of this phenomenon is the continuous production of new breaks detectable from formation of chromatid bridges for many generations during the multiplication of the cells. This finding suggests that the phenomenon is due to persistence in the cells of an agent, possibly related to polyoma virus, even when infectious virus is no longer present. The production of chromatid breaks is not, however, a necessary consequence of the interaction of the cells with this virus since it is absent in transformed cells deriving from the permanent hamster BHK line. The reason for this difference is not clear.

Cell Transformation

Many tumor-producing viruses induce a hereditary change in the cells after infection called transformation; the transformed cells behave as cancer cells in the animal. The aspect of transformation to be discussed here is the nature of the interaction of viral DNA with cellular genetic material; we shall limit our consideration to polyoma virus, although similar phenomena may be caused by other viruses, such as SV40 or the carcinogenic adenoviruses.

Since transformation of mouse or hamster cells is a hereditary change in the cells induced by viral DNA, it is relevant to inquire whether it is caused by a phenomenon similar to lysogenization (Dulbecco, 1964). Extensive experimentation has shown that the phenomenon differs from

lysogenization since induction does not occur and there is no evidence for the production of an immunity repressor; it could be similar, however, to the insertion of a defective prophage which lacks both nuclease and repressor genes. Such inserted viral DNA could be detected functionally, i.e., by identifying certain gene functions, or genetically, i.e., by rescuing certain genes after infecting the transformed cells with genetically marked polyoma virus, or physically, i.e., by demonstrating DNA homologous to polyoma DNA in the DNA of the transformed cells by hybridization experiments. Evidence of the first and second types has been obtained (Sjögren *et al.*, 1961; Habel, 1961; Ting, 1964) although it is still questionable whether the evidence conclusively shows that genes of the virus are present in the transformed cells; we shall consider it acceptable. Physical demonstration of the presence of viral DNA has not been convincingly obtained, and it seems likely that it cannot be obtained owing to lack of sensitivity of the methods if there are only one or two copies of viral DNA per cell (Winocour, 1965). Homology of viral DNA for DNA of normal mouse cells has been detected (Axelrod *et al.*, 1964). This homology, however, could be spurious, and caused by contaminating mouse DNA in the preparations of polyoma virus DNA; if genuine, it may indicate a relationship between polyoma virus and mouse cell DNA's similar to that between λ and *Escherichia coli* DNA's. It is not clear whether this homology, if genuine, plays a role in transformation, since no homology has been detected between polyoma DNA and hamster cell DNA although hamster cells can also be transformed by the virus.

An interesting similarity between the DNA of polyoma virus and the DNA of λ is the ring shape of the molecules (Dulbecco and Vogt, 1963; Weil and Vinograd, 1963). During intracellular reproduction, polyoma DNA is in a circular shape most of the time and thus could become inserted into the cellular DNA like λ DNA. Another similarity between the two DNA's can be detected in a fraction of DNA extracted from polyoma virus which is devoid of infectivity, differs in structure and base composition from the regular polyoma DNA, and has a different buoyant density (component III, Weil and Vinograd, 1963). This DNA component has not yet been shown to be genuine polyoma DNA; if this is the case, it may arise by a mechanism similar to that giving rise to the formation of the defective *λdg*.

The reported observations show that an anatomical relationship between the DNA of polyoma virus and that of the transformed cells is strongly suspected, but not conclusively proved. A functional relationship,

on the contrary, is well documented. Infection with polyoma virus of resting cell cultures in which very few cells synthesize DNA causes a marked enhancement of synthesis. After a lag period of 8 to 10 hours, both the synthesis of DNA and that of a group of enzymes related to DNA synthesis start in the cultures at a high rate (Dulbecco *et al.*, 1965). The DNA synthesized is partly viral and partly cellular; the enzymes are specified by cellular genes. Thus, polyoma virus affects the regulation of a group of cellular genes involved in DNA synthesis which is referred to as the DNA complex. Induction of these new syntheses appears to follow the synthesis of another protein, presumably a regulatory protein. Since the DNA complex of uninfected cells is similarly regulated, the regulatory protein formed after infection may be specified by a cellular gene which has the role of regulator of the DNA complex.

These results show that the mechanism by which polyoma virus affects the function of cellular genes is complex, probably much more so than bacterial regulation. It seems likely that these differences are related to the different regulatory organization of bacterial and animal cells. Animal cells have a mechanism which regulates the rate of cell multiplication depending on information coming from the environment, in part through cell contacts; such a type of regulation is absent in bacteria. Thus, in animal cells the surface membrane has the role of a sensor which receives information from the environment and transmits it to a regulator gene, presumably the regulator of the DNA complex. A function of the viral DNA interferes with this process of information transfer; the interference may well occur at the level of the cell surface which in the transformed cells develops characteristic antigenic changes.

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THE REPRODUCTION OF SUBCELLULAR SYSTEMS

Chromosome Reproduction in Mitosis and Meiosis

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Introduction

The problem of chromosome reproduction is an old and honorable one, but it retains its challenging freshness because of its pertinence to our understanding of the role of the cell in growth and development, and because the newer techniques of microscopy, enzyme digestion, labeling, and whole chromosome isolation continue to offer intriguing hopes of solution. If we restrict our attention to the chromosomes of eucells as we shall here, we realize immediately that the problem of reproduction is neither circumscribed by, nor synonymous with, DNA replication, although the two processes are coincident, or very nearly so, in time. DNA replication is of course, a crucial part of chromosome reproduction, and it may well be, as Prescott (1964) argues, that all other elements in the chromosome are indentured to DNA. The relative ease with which the replicative process may be followed experimentally, however, should not blind us to the fact that the chromosome is more than a strand of DNA and that the other molecular species, which must also be formed and put into place, are necessary to the chromosome in its role as a maneuverable, functioning organelle of the cell. Furthermore, as Mazia (1961) has discussed at length, and as Stebbins (1950) points out in his consideration of aberrant meiosis in apomictic plants, the proper progression and tempo of events in chromosome reproduction are as important to the final success of cell division as is the proper completion of each individual reproductive event. In focusing our attention on chromosome reproduction, therefore, our object is to view it as an integral part of the more complex phenomenon of cell division, and to search for those correlative events which lead to and stem from chromosome reproduction.

The following discussion will be couched in general terms, avoiding

experimental detail where possible, and will be concerned with three aspects of the problem. We will first consider replication as a process; then we will examine the reproduction of the chromosome as an organelle from the point of view of functional and genetic necessity; finally, using the meiotic cell as a model, we will consider the consequences of chromosome reproduction on those cellular events which distinguish the meiotic from the mitotic cell. Reference should be made to Moses and Coleman (1964) for the most recent and detailed review of chromosome function and organization.

Replication As a Process

Throughout the following discussion, we have adopted the sense of replication defined by Pollock and Mandelstam (1958); that is, as "polyvalent autocatalysis . . . there is freedom of copy, i.e., the *same* system has the potential ability to copy more than one species of prototype molecule, and what it actually produces is determined by the prototype which initiates the process." In the chromosomes of eucells then, we may refer with propriety only to DNA as a replicating species; other constituents are dependent upon heterocatalytic activity. Even intimately associated molecular species such as histones must be synthesized by derivative action of the prototype system.

Since our discussion is focused upon chromosomes, we are led to the conclusion that linearity of structure is a necessary (but hardly sufficient) prerequisite for replication; that is, biological structures of a nonlinear kind are synthesized but not replicated. We must clearly reserve judgment on the extension of the principle to centrioles, plastids and mitochondria until clarification of the mode of reproduction of their non-nucleic acid components has been achieved. The chromosome of the eucell, however, is a sensibly linear entity which has a replicating component derived from a limited assortment of nucleotides produced by prior synthetic events. The *selection* of the appropriate molecules from the pool of varied precursor substances is, as Muller pointed out as early as 1929, a significant and critical aspect of replicating systems. With rare exceptions (and such systems are indeed mutable), the prototype and its enzymatic apparatus select only the appropriate nucleotide elements suited to the reproducing system.

For replication (and subsequent heterocatalysis) to occur a number of predictable, and to a certain extent experimentally detectable, events must take place in an otherwise competent cellular environment. Among

these are development of the nucleotide pool for DNA and RNA, development of the amino acid pool for selection and incorporation into chromosomal proteins, and activation (release?) or induction of the pertinent enzymes. In addition, the prototype, DNA, must be brought to an activated state, presumably by opening up the double helix (Sibatani and Hiai, 1964); (however, see Cavalieri and Rosenberg, 1961, for conservative replication of DNA). As a special act of synthesis in eucells, replication enjoys precise temporal limits with decisive initiating and terminating points. Parenthetically, it can be pointed out that in an evolutionary sense the decisive termination of replication is a necessary feature in multicellular organisms if differentiation is to take place.

Although its regulation displays reproducible variation, replication nevertheless seems subject to firm biological control. It is equally evident that the process must come to a close by some equivalent mechanism involving exhaustion of substrate, enzyme inactivation or destruction, prototype deactivation, or some other controlling event(s) presently unknown. These matters have been discussed in detail by Mazia (1961, 1963), Stern (1962), and Taylor (1963), and no further comment is warranted here other than to point out that no single mitotic or meiotic "trigger" for initiation of the reproductive process has been identified nor, according to Stern, should one be expected any more than one is expected for any other regulated or induced event in the life of the cell. Though perhaps less evident, this same stricture holds for the termination of the replication events.

Replication of DNA

It is usually assumed that the incorporation of radioactive thymidine into the chromosome implies the occurrence of the replication process. Conceivably, a sequential two-step process could be involved, i.e., the assimilation of the nucleotide by the chromosome and its incorporation into the growing polymer may not be simultaneous processes, but in the absence of meaningful evidence to the contrary they can be assumed to constitute a single event taking place during the S period of interphase. In eucells at least, and if the nucleus as a whole is considered, the S period is sharply limited in time. It may be at the beginning, middle, or end of interphase, or it may, as in *Tetrahymena* (Walker and Mitchison, 1957) and grasshopper neuroblasts (Gaulden, 1956), occupy all or nearly all of the entire interphase period; as a rule, however, it is preceded and followed by nonsynthetic periods (the

so-called G_1 and G_2 stages of interphase). In relation to the cell cycle as a whole, the S period occupies roughly 35–40% of the time span in distinct contrast to the circumstances taking place in a bacterial cell during the exponential phase of growth. Here less than 10% of the cell cycle is in a nonsynthesizing DNA state (Pachler *et al.*, 1965), and Oishi *et al.* (1964) have provided evidence that one round of replication may be initiated in the bacterial chromosome before the preceding round has been completed.

The initiation of DNA synthesis is preceded by protein formation, and this presumably is preceded by the formation of RNA derived by transcription from a selectively activated portion of the genome (Maaløe and Hanawalt, 1961; Stern and Hotta, 1963). The controls for this apparently inductive system are unknown, although it is possible that the migratory proteins described by Goldstein (1963) and Prescott (1963) in ameba exercise a regulatory action of this kind. On the other hand, proteins formed prior to DNA replication cannot be considered mitotic or meiotic triggers which set off a sequence of events necessarily culminating in cell division. As Stone and Prescott (1964) have shown, DNA synthesis can be initiated but not completed in the absence of essential amino acids. DNA replication and cell division, although sequential in normally dividing cells, are distinct and separable cellular events, each of which can take place without the other. DNA replication alone leads to polyploidy and/or polyteny, both commonly observed phenomena. Cell division without DNA replication has been observed in synchronized cultures of *Tetrahymena* (Zeuthen, 1963, 1964) and must be presumed to occur in the cells of the hind gut of the mosquito where somatic reductions regularly take place during metamorphosis (Berger, 1938; Grell, 1946).

The duration of time over which DNA and the chromosome replicate can vary from a matter of seconds in bacteria to many days in higher organisms. If a semiconservative mode of replication is assumed, DNA cannot replicate faster than the single polynucleotide strands can unwind, and unwinding time increases as the square of the molecular weight of the uninterrupted DNA strand (Freese and Freese, 1963). According to the calculations of Freese and Freese, the minimum time required for a T2 phage chromosome to replicate would be about 7 seconds. A bacterial chromosome consisting of a single uninterrupted double helix (as indicated by electron microscope evidence) could complete its replicative process in about 30 minutes; this is a rate of about 20–30 μ

per minute. Chromosomes of eucells, however, require time of a different order of magnitude. This is suggested by the amount of calculated unwinding time (Freese and Freese, 1963, Table III), and is borne out by actual observations. We shall return to this problem later because of other considerations, but a casual view of replication times in different eucells indicates that while the S period occupies roughly 40% of the cell cycle, the actual duration in measured time can vary considerably.

Karyokinesis in *Drosophila* embryos takes place in less than 15 minutes. The portion of the cycle taken up by DNA synthesis is not known, but it must be relatively brief. *Vicia* roottip cells, on the other hand, show thymidine incorporation for a period of 7.5 hours out of a total of 19.3 hours (Evans and Scott, 1964), and a roughly comparable figure of 6 hours out of about 18 hours total holds for human and hamster cells (Hsu *et al.*, 1964). A time closer to 15 hours for DNA synthesis is found in the microspores of cultured lily anthers (Stern and Hotta, 1963, Fig. 3), and it would be of interest to know whether this slower pattern is characteristic of the lily or peculiar to the microspores themselves. However, the very different rates of cell division in embryonic and adult tissues suggest that control of the rate of replication is tissue- as well as species-regulated.

A comparison of rates of synthesis in mitotic and meiotic cells has been obtained in the grasshopper (Muckenthaler, 1965). The S period of spermatogonial cells is of 12 hours duration out of a total of 28 for the entire cell cycle. The S period in meiocytes, on the other hand, is at a much slower tempo—9 to 10 days out of a total of 28—but once again the regulatory control is unknown even though the percentage of the cell cycle occupied by the S period is close to the 40% average.

The extent of the S period has made it possible to show that this period in eucells is not only decisively initiated and terminated, but that an internal asynchrony of synthesis of a reproducible nature is characteristic. Regulation, therefore, must operate intra- and interchromosomally as well as for the nucleus as a whole. The chromosome does not simply begin to replicate at one end and proceed to the other end as might be expected of a single DNA double helix, nor do all chromosomes replicate simultaneously. This theory was first demonstrated for the X chromosome of the grasshopper (Lima-de-Faria, 1959). This chromosome, as well as heterochromatin in general and euchromatin that has been heterochromatinized through differentiation (to give the Lyon effect), is late replicating, but euchromatin can also exhibit varying

temporal patterns of synthesis. German (1964), in fact, has been able to characterize many chromosomes of the human complement by their patterns of replication, as have Hsu *et al.* (1964) in the hamster.

These facts return us to the considerations of Freese and Freese (1963) concerning the time required for the replicative process. As they point out, the minimal time of unwinding of the DNA double helix is a function of the square of the molecular weight of the DNA strand. If it is assumed that the DNA in a single human chromosome of average length is in the form of an uninterrupted double helix which must unwind in order to replicate, 400 hours would be the minimal replicative time (Freese and Freese, 1963, Table III). Six hours is known to be more reasonably accurate. A comparable chromosome in *Vicia* would require 2×10^5 hours instead of the 7.5 hours indicated by Evans and Scott (1964). Consequently, Freese and Freese state unequivocally that large chromosomes such as those found in eucells cannot contain all of their DNA in uninterrupted strands, at least not throughout the period of replication. The data of Plaut and Nash (1964) would seem to support this point of view. They have demonstrated that in the X chromosome of *Drosophila* salivary glands a minimum of 50 points of discrete DNA precursor incorporation are present, a situation that indicates the presence of that number of "free ends," and suggests further that the integrity of the chromosome is not dependent upon a continuous and uninterrupted DNA strand. However, Plaut and Nash (1964) caution, as do Freese and Freese (1963), that discontinuous labeling implies discontinuity of DNA strands only during the period of replication; the structural nature of the chromosome at other times during the cell cycle cannot necessarily be deduced from these observations.

The replication of DNA in the salivary chromosomes of *Drosophila* presents an interesting problem in mechanics. Freese and Freese (1963) base their considerations of unwinding time on an unrestricted rotation of the DNA molecule, but the growing salivary chromosome in its stretched and polytene condition appears to impose rather severe restrictions on such movement. Whether the dilemma is real or imagined cannot be decided without further knowledge of the arrangement of molecules in the giant chromosomes and their impedance to unwinding under such conditions.

The discrepancy among replication times, i.e., the 6 hours of actual time according to Hsu *et al.* (1964) and the calculated 400 hours of Freese and Freese (1963), might be resolvable if the degree of stranded-

ness is taken into account. If it is assumed that the chromosome consists not of one continuous or tandemly arranged DNA strand, but of 64 lateral strands of shorter length, then the difference between actual and calculated times of replication becomes negligible. If, on the other hand, the number of replicons in such chromosomes approaches 64, as Plaut and Nash (1964) suggest in the case of the *Drosophila* chromosome, the time discrepancy is also resolved, with only one or two helices per chromatid being necessarily present. Obviously our ignorance of chromosome structure prevents us from further profitable speculation, at least from speculation based on these data.

The point has been made that the synthesis of DNA in the chromosomes of eucells is sharply restricted in time, even though within a given genome the process exhibits more or less characteristic patterns of asynchrony. A number of early reports (Ansley, 1954; Moses and Taylor, 1955) indicated that such synthesis may extend into the early stages of prophase, when presumably the chromosomes had already entered the beginning of a contraction phase; but since these data were derived from spectrophotometric rather than labeling studies, they may with reason be viewed with some suspicion. At least two indications of prophase incorporation of DNA precursors, however, are worthy of note. Wimber and Prensky (1963) have indicated that spot labeling appears to take place during meiotic prophase (pachynema), and suggest that this may be related to problems of crossing-over. Their photographs show an even distribution of grains over the chromosomes with no concentration of grains associated with the distribution of chiasmata. It would be of interest to pursue this problem further in organisms with sharply restricted chiasma distributions (Mather, 1938), or in some organism such as the male mantid in which crossing-over and chiasma formation are absent.

McGrath (1963) has also provided evidence to show that grasshopper neuroblast chromosomes incorporate thymidine in prophase after exposure to X rays. Such cells tend to revert to earlier stages after exposure to X rays, and the greater the degree of reversion, the greater the degree of incorporation of the label. Whether this phenomenon relates to the repair of X-ray induced breaks in the chromosomes through the action of a "healing enzyme" is problematical, but the fact that incorporation does occur indicates that the mechanism for incorporation is present in prophase, and that partially condensed chromosomes are not wholly incapable of DNA precursor uptake. It is well to recall here Cleveland's (1949) study on the flagellated protozoa. These or-

ganisms possess no recognizable interphase; the chromosomes are visible at all times and hence are always contracted to a certain extent, yet they must reproduce while contracted. In contrast, Stone *et al.* (1964) show that in *Tetrahymena,* labeled thymidine is taken up only during the S period even though a pool of thymidine derivatives persists from the end of one period of DNA synthesis to the beginning of the next. The pool does not turn over during non-S periods. McGrath's (1963) evidence suggests that if such a pool exists in neuroblast nuclei, X-rays activate the pool and permit the entry of labeled thymidine.

Formation of Other Chromosomal Components

Prescott and Bender (1963a) and Prescott (1963) have stated that DNA is the only permanently conserved molecular component of the chromosomes of higher organisms. The replication of DNA is therefore of crucial importance in the life cycle of chromosomes, but few cytologists would dispute the statement that the chromosome, structurally and behaviorally, is substantially more than just an unknown number of DNA molecules. Regardless of the degree of stability of the proteins and RNA of the chromosome, these molecules must play a significant role, and their formation is of necessity a part of chromosomal reproduction.

The histones have been the recipients of adulatory attention during the past few years, and apparently with good reason (Bonner and Ts'o, 1964). Nonetheless, they remain elusive, enigmatic substances in regard to diversity of structure, role in cellular metabolism, and relation to chromosomal structure and behavior. Only the latter point will be discussed here.

The histones cannot replicate in the same sense that DNA can; their structure, of course, does not permit the formation of complementary amino acid polymers analogous to polynucleotide chains. They are, furthermore, segregated in a dispersive manner (Prescott and Bender, 1963b; Prescott, 1963), which again suggests their relative impermanence as a substantive portion of the chromosome. On the other hand, the time and duration of their synthetic period generally coincides with that of DNA in interphase (Bloch and Godman, 1955; Ansley, 1957; McLeish, 1959; Umana *et al.*, 1964). However, protein synthesis, together with that of RNA, proceeds well into prophase (Hotta and Stern, 1963; Das *et al.*, 1964). A conclusion which can possibly be drawn from these studies is that the later formed proteins are not histones, but rather con-

stitute the products of genes concerned with the sequential events of cell division up to the conclusion of the process. If this is so, then some sites, presumably within the coiled regions of the chromosome, must be capable of transcription. Only metaphase and anaphase chromosomes appear to be wholly inactive metabolically (Prescott, 1963).

The relation of histones and their contribution to a changing chromosomal morphology is not clear. An equimolar amount of histone is thought to be complexed with DNA (*cf.* Zubay, 1964), although Umana *et al.* (1964) provide data which reveal that the 1:1 histone: DNA ratio is characteristic only of dividing cells. Considerably higher ratios of varying values were obtained from tissues in which the majority of cells were in interphase. Nevertheless, the structural relation of histone to DNA is still uncertain. Zubay and Doty (1959) suggested that the histones followed the larger groove in the DNA helix. More recent X-ray diffraction patterns require a modification of this hypothesis, and Zubay (1964) now believes it more likely that, at least in the gel state, the histones form a sheet-like structure bridging either parallel DNA helices or successive coils of a single helix. The long axes of the histone molecules parallel the large groove of DNA, and Zubay (1964) postulates that there may be a transition during sol to gel transformation of the DNA which causes the histone to shift its position from the large groove to the sheet-like arrangement.

The conclusion seems inescapable that histone, apart from its suspected regulatory action on genetic material, plays a role in the contraction of chromosomes and in maintaining the stability of DNA as a molecule. The contractile role is suggested by the fact that DNA-histone particles *in vitro* double their length when the histone is removed (Marmur and Doty, 1961). On the other hand, the histones possess substantial molecular diversity (Busch *et al.*, 1963, 1964); there is no information as to the respective roles of the different histones in chromosome contractility and morphology. Trosko and Wolff (1965), for example, on the basis of pepsin digestion of air-dried *Vicia* metaphase chromosomes (a procedure which renders them alkaline fast green negative) found that the removal of histones does not alter chromosome morphology, and advanced the thesis that these molecules have little to do with contractility.

An understanding of the nonhistone or acidic proteins—chromosomin or residual protein—both as to their time of formation and their role in chromosome function and morphology, is even more fragmentary than it is for the histones (Busch *et al.*, 1963, 1964). Consequently, specu-

lation endows them with various roles, metabolic and structural. Recent enzymatic studies (Trosko and Wolff, 1965) support earlier suggestions that the acidic proteins are responsible for maintaining the linear continuity of the chromosome, a hypothesis in keeping with the latest chromosome model of De (1964). Once again, however, the investigations of Prescott and Bender (1963b) and Prescott (1963, 1964) leave little doubt that any protein, basic or acidic, can be considered a "conserved" element of the chromosome for more than three or four divisions.

Chromosomal RNA is probably an even more transient molecular species (Prescott and Bender, 1963b; Prescott, 1963, 1964) although it is regularly associated with chromatin at all stages of the cell cycle (Edström, 1964). It is undoubtedly DNA-derived, may be present in varying concentrations and base compositions depending upon the portion of the chromosome from which it is extracted (Edström and Gall, 1963; Edström, 1964), and is destined in all likelihood for cytoplasmic sites to play a part in protein synthesis. It does not appear to be involved in a determination of chromosome morphology or reproduction in the sense of being an integral element, since RNase does not alter chromosomal morphology (Trosko and Wolff, 1965). Its role, therefore, appears to be metabolic, not structural.

Chromosome Reproduction

Chromosome reproduction involves the formation, by replication or synthesis, of all molecular species which give the chromosome form, regulate its metabolic activity, and enable it to maneuver during the cell cycle. DNA is the only permanently conserved molecule found in the chromosome; by comparison, the proteins and RNA are transitory. However, when we view the reproduction of chromosomes in a structural sense, we obviously cannot equate this phenomenon with the replication of DNA alone. We recognize that the DNA of the chromosome is segregated semiconservatively in both mitosis and meiosis, and by inference we assume that this follows because DNA is similarly replicated, although Cavalieri and Rosenberg (1961) argue otherwise. The same argument cannot be advanced for the proteins and RNA of the chromosome. The latter molecule is a transcriptional element that apparently plays no part in chromosome morphology; its position in metaphase chromosomes is that of a peripherally bound molecule (Prescott and Bender, 1963b). The proteins are dispersively distributed, but they

are more intimately bound to the chromosome than is RNA. The studies of Goldstein (1963) and Prescott (1963, 1964) have revealed the presence of a migratory protein that moves in and out of the nucleus during each division cycle, but the relation of this protein to general cellular activity remains unknown. Its role in chromosome reproduction may well be central, although its role in determining chromosome morphology (at least of the metaphase and anaphase chromosome) is negligible.

The confirmation of Taylor's early labeling studies by Prescott and Bender (1963a), and carried by the latter through four cell cycles, leaves little doubt about the semiconservative segregation of DNA (see also Simon, 1961; Walen, 1965). To explain these results it is necessary to recall that the morphological unit of segregation is the chromatid, and that it is also the unit of coiling and crossing-over. What then is the unit of replication? Granted, at the molecular level DNA is replicating, but does the DNA of the interphase chromosome at the time of synthesis exist as a single double helix or as some multiple thereof? It is so evident from the cytological literature that this question has been posed many times and no satisfactory answer given, yet the experimental results demand that some unit of the chromosome must behave in singular fashion prior to the S period, as though it were double after DNA synthesis and never, except possibly in polytene chromosomes, in any larger multiple of two.

The simplest hypothesis is the assumption that, prior to replication in interphase, the chromosome consists of a single double helix of DNA with its associated proteins. Taylor (1963) subscribes to this point of view which is supported by several experimental studies on amphibian lampbrush chromosomes (Callan, 1963; Gall, 1963). These are meiotic chromosomes, but the pattern of DNA synthesis in the S period, extended over a longer period of time, does not appear to differ quantitatively or qualitatively from that in a mitotic cell. Furthermore, and despite the fact that lampbrush chromosomes are the longest chromosomes of which we have knowledge, they arise from a contracted state and at a later time return to this contracted state. Their changing length and width, therefore, are functions of the degree of coiling or uncoiling since no additional round of DNA synthesis takes place during their long period of development. The lateral loops of these chromosomes have been shown, through stretching experiments (Callan, 1963), to be part of the linear continuity of the chromosome, to contain DNA, and to possess a diameter of about 40 Å. The loops are part

of the length of a whole chromatid, not of some lesser lateral subdivision. Since they can be digested only by DNase there can be little doubt that the chromatid of a lampbrush chromosome contains only a single double helix of DNA. The single double helix is, therefore, the unit of replication as well as the unit of segregation, coiling, and crossing-over.

This point of view is supported by quantitative considerations (Callan, 1963; Gall, 1959). A close correspondence should be found between the total amount of DNA and the calculated length of the chromosomes in a haploid complement. Any discrepancy between these two sets of figures should be a reflection of the degree of lateral redundancy of DNA double helices. In two species of newts, *Triturus viridescens* and *T. cristatus,* the total length of the chromatids, counting loop lengths, is of the order of 50 cm. Since only one-twentieth of the loops are evident at any one time, the total length of DNA in the haploid complement is approximately 10m. Considered in terms of the total DNA in a haploid complement, 3×10^{-5} μg, the conclusion again seems inescapable that a chromatid possesses only a single double helix of DNA.

The argument presented here does not presuppose or demand that the DNA is uninterruptedly continuous from one end of the chromatid to the other. Indeed, the mechanical considerations of unwinding (Freese and Freese, 1963) and the existence of numerous foci of discrete and simultaneous labeling (Plaut and Nash, 1964) suggest but do not prove that, during synthesis at least, DNA is discontinuous, i.e., the chromosome behaves in replication like a number of more or less independent replicons (Jacob *et al.,* 1963). The viability of homozygous inversions and translocations in many organisms provides additional support of a different kind for the theory that break points can occur between rather than within structural genes. Whether linker molecules are present, or in their absence, what keeps separate replicons in perfect tandem array, genetically as well as structurally, remain as unsolved questions.

In contrast, Read (1961) and Steffensen (1961) have carried out calculations similar to those of Gall and Callan, but based on the lengths of mitotic and meiotic chromosomes of more conventional size. Both arrive at a figure of 200 to 300 for the number of lateral subdivisions in the chromatid, but the difference in conclusions can be attributed largely to different basic assumptions regarding the length of the DNA molecule when fully extended. The hazards of estimating, rather than directly measuring, length are obvious when it is realized that a minimum

of four orders of coils exist in a normal metaphase chromosome (Inoué, 1964).

The single double helix per chromatid hypothesis is attractive for a number of reasons: it agrees with the extraordinary exactness of chromatid segregation in mitosis and meiosis, and it is in accord with biological economy achieved through natural selection since excessive redundancy of genetic material is eliminated. If we invoke the principle of parsimony, it would not be unreasonable to suppose that the minimum and maximum number of double helices per chromatid would be the same. The unit of replication becomes synonymous with the unit of segregation, coiling, and crossing-over. There are, however, a variety of observations and interpretations which cannot be summarily dismissed; the first of these is the fibril revealed by electron microscopy, its nature, and its relation to the chromatid as a whole. Sectioned nuclei and isolated whole-mounted chromosomes reveal the presence of numerous fibrils longitudinally oriented in the long axis of the chromatid. Ris (1962), Kaufmann *et al.* (1960), and others have interpreted these fibrils as linear and parallel components of the chromatid, and therefore, suggest that the chromatid possesses a polytene structure. The basic fibril has a diameter of about 100 Å, which is resolvable in favorable preparations into two 40 Å strands apparently plectonemically intertwined about each other. The 40 Å unit has been equated with the DNA double helix and its associated proteins. Ris (1961), in fact, estimates that the post-replicative leptotene chromosome of *Tradescantia* contains thirty-two 100 Å fibrils, or sixty-four of the 40 Å units. This interpretation has most recently been disputed by Hyde (1964), who subscribes to the simpler hypothesis of one helix per chromatid. The fibrils may, in fact, represent successive foldings of one, or at most a few, long molecules.

Radiation studies provide another source of disagreement, although not to the extreme extent of the polytenist hypothesis. Half-chromatid aberrations, induced by X rays and ultraviolet radiation, have been reported by a number of investigators (Swanson, 1947; Sax and King, 1955; Wilson *et al.*, 1960; Crouse, 1961). The findings of Sax and King are of particular interest. Irradiation of *Tradescantia* microspores leads to the induction of chromosome, chromatid, and half-chromatid aberrations, and the appearance of each is dependent upon the time of irradiation in relation to the mitotic cycle. Chromosome aberrations are induced when irradiation precedes DNA synthesis (however, see Evans

and Savage, 1963), chromatid aberrations in the post-replicative G_2 period. The appearance of half-chromatid aberrations following irradiation in prophase (post-pachytene in meiotic cells) poses a problem, therefore, since no new round of DNA synthesis has taken place. Why the chromatid behaves single to X rays in one instance and double in another is not clear. Are these visibly evident aberrations, often markedly extended in meiotic cells (Crouse, 1961), a double helix in a coiled state, or only a single polynucleotide strand? Crouse adopts the latter point of view, but the diameter of the strands provides no answer since the degree of coiling is unknown. The data of Peacock (1963), based on isolabeling of X_2 metaphase chromosomes, suggest that prior to replication the chromatid consists of two helices instead of one, an interpretation in keeping with the induction of half-chromatid aberrations. The existence of half-chromatid aberrations, on the other hand, has been challenged by Ostergren and Wakonig (1954), who argue with reason that half-chromatid aberrations found in the first (X_1) metaphase after irradiation should show up as chromatid aberrations in the subsequent (X_2) metaphase. Making use of colchicine to induce X_2 tetraploid cells, they have been unable to find aberrations which they could refer back to the half-chromatid aberrations of the preceding metaphase. We have repeated this experiment, making use of 5-aminouracil to obtain synchronized divisions, but our results to date are basically in accord with those of Ostergren and Wakonig (1954).

A third source of disagreement with the one helix per chromatid hypothesis are the observations made with light microscopy. Among others, Manton's (1945) ultraviolet photographs of *Todea* (a fern) anaphase chromosomes are highly suggestive of a multistranded structure, as are those of Hughes-Schrader (1940) in the coccid, *Llaveiella,* and those of Trosko and Wolff (1965) on the enzymatically relaxed metaphase chromosomes of *Vicia*. The latter study, if coupled with autoradiography, could be crucial in the resolution of the nature of the bifurcating fibrils. In fact, until this question is settled at both light and electron microscope levels of resolution, the problem of chromosome reproduction, as distinct from DNA replication, remains with us. We can only state, in summary, that some as yet unidentified element of the chromosome must reproduce in such a manner that the chromosome can segregate in a semiconservative fashion. If the chromatid consists of a single double helix, this element could be DNA; if the chromatid is multistranded, then some other molecular species is involved which is not only semiconservatively segregated itself, but is so structured to

DNA that many laterally arranged DNA molecules behave in a unitary fashion. The chromosome model offered recently by Wolff (1965) was proposed in order to overcome the difficulties just described, but the relation of the model, based on the multistranded concept, to chromosomal reality remains uncertain.

Consequences of Chromosome Reproduction

The events of replication double the complement of chromosomal materials in the cell; the unit replicated, at least in a genetic sense, is the chromatid. The consequences when cell division ensues are an increase in cell number (with genetic identity) in mitosis, or a reduction in chromosome number (with genetic recombination) in meiosis. Chromosome reproduction, however, is not a necessary event in the life of a cell; it has genetic significance only when projected into the future, and hence is accompanied by cell division. Repeated replication without cell division leads, of course, to endopolyploidy or polyteny, but we have little understanding of these reproductive events in the chromosome and their relation to the concurrent or subsequent behavior of the cell. At the most superficial level of understanding, repeated replication without cell division indicates a disengagement of the coordinate regulation of replication and cytokinesis. Other cells such as the nucleated red cell of birds undergo no further nuclear change (Cameron and Prescott, 1963), and it would be of interest to know to what extent cells scheduled for immediate differentiation do or do not undergo DNA replication. The point is that the events of mitosis and meiosis, including chromosome reproduction, are dissociable from each other even though under ordinary circumstances they are coordinated, in parallel or in sequence. It has been demonstrated in *Tetrahymena* (for example, Zeuthen, 1964) that synchronization procedures readily alter the processes of cell division without any appreciable effect on the rate of DNA replication, but it is equally clear that in the cells of multicellular plants and animals a high degree of regularity of events is the customary pattern, although the separate processes may be individually blocked or altered (Stern, 1962; Mazia, 1961). We may then inquire whether this degree of regularity demands a parallel degree of dependence of one phase of division on another, a question prompted in part by the common knowledge that any agent inhibiting DNA synthesis also inhibits cell division, although the contrary is not true.

Complete inhibition of both DNA replication and cell division tells

us little of the process. Unfortunately, only a limited number of studies on the effect of partially inhibiting the process(es) are available. This work indicates, however, that the consequences are recognizable as errors in the phasing of events. Stubblefield (1964), for example, has shown that late replicating segments of hamster chromosomes entering G_2 prior to the apparent completion of DNA synthesis, fail to contract in normal fashion, and reach the metaphase stage in an extended "prophase" stage. On the assumption that the proteins of the chromosome aid in contraction, this would suggest that a completed replication is a necessary condition for successful protein attachment. These findings recall the earlier studies of Darlington and La Cour (1938, 1940) on the differential stainability of chromosomes induced by prolonged cold treatment. They referred to the phenomenon as "nucleic acid starvation," a view generally rejected with an argument for constant DNA value per chromosome complement. Wilson and Boothroyd (1944) preferred to view the phenomenon as an example of differential coiling, a position expanded upon by Ris (1945) who expressed the opinion that any linear differentiation of the chromosome is a function of local coiling patterns. It would now appear that these are not alternative explanations, but that each is a partially correct description of a situation in which coiling is dependent on the amount of DNA. La Cour *et al.* (1956) have shown spectrophotometrically that nuclei having differential stainability do possess a lowered DNA value.

That protein is involved in the contraction of chromosomes is also suggested by the work of Stern and Hotta (1963). When protein synthesis is blocked by selective inhibitors, chromosome morphology is affected (the chromosomes are less contracted) without any effect on segregation at anaphase. A comparable genetic situation in maize has been described by Rhoades (1956) in which a gene (*el*) modifies the meiotic chromosomes in such a manner that they arrive at M_I and M_{II} in an elongated state. Again, segregation is not disturbed, but it would be of interest to know whether the protein content of these chromosomes, quantitatively, or structurally, is in any way shifted from normality. The functional integrity and normal behavior of the centromere, at any rate, is maintained.

Another molecular species given very little attention as a chromosomal component are the phospholipids. La Cour and Chayen (1958) point out that contraction of the chromosome and uptake of phospholipids coincide in time, but whether the correspondence in time is fortuitous or not is unknown.

A problem worthy of reconsideration in relation to chromosome reproduction is the old one of nondisjunction. Usually viewed as an accident of segregation brought on, at least in *Drosophila,* by failure of crossing-over, nondisjunction is a regular feature in certain species [rye, Muntzing (1946); maize, Rhoades (1952)]. In these two species, the accessory chromosomes fail to disjoin at the first and second microspore divisions, respectively. The centromeres of these chromosomes as well as their ends are clearly separate from each other, but the chromatin adjacent to the centromeres fails to separate cleanly. Is this due to failure of completed replication, or to delayed replication and abnormal protein attachment? The problem should be accessible through the newer labeling techniques.

A third problem to be considered here is the relation of chromosome reproduction to synapsis in meiotic cells. Ansley (1954, 1957) has indicated that disturbed DNA: histone ratios are associated with asynapsis, suggesting that these two molecular species, normally synthesized nearly together and in equal amounts, must be in the proper proportion if the events of meiosis are to follow in their normal sequence. The ready availability of asynaptic forms in both plants and animals should permit a further penetration into what has continued to be an enigma of chromosome behavior. The genetic anarchy to which this form of misregulation leads is evident.

Last, we wish to make a comparison between mitosis and meiosis, not so much from considerations of chromosome reproduction—for we see no immediate relevancy—but mainly in relation to the problem of differentiation. Stern (1962), in his provocative review of chromosome structure and function, has advanced the thesis that "the whole phenomenon of chromosome reproduction can be defined as a special instance of cell differentiation in which induced changes are not stabilized but are transient and recurrent." When this theory is applied only to the problem of chromosome reproduction we find no cause for disagreement. Except for the longer duration of the S period in meiosis, there is little to distinguish between the reproductive events occurring in mitotic and meiotic chromosomes. Whether this longer synthetic period of meiosis is responsible for, or permits, the subsequent events of synapsis and crossing-over is not known, although the "retardation" hypothesis of Beasley (1938) is predicated on this basis. Furthermore, as Stebbins (1950) points out, apomictic plant species in which meiosis is greatly disturbed are generally characterized by an altered pace of meiotic events; they are either much retarded or greatly speeded up.

A proper timing of events is important. However, we believe that emphasis should be given to the fact that although mitotic and meiotic cells pass through a similar cycle of chromosome reproduction, they possess quite different characteristics. In the mitotic cell, differentiation (used in the conventional sense) *follows* cell division; the literature on growth and development suggests that these are mutually exclusive processes. The meiotic cell, on the other hand, *is* a differentiated cell. Once formed, it is irrevocably committed to a given course of action. Any of the events of meiosis—synapsis, crossing-over and chiasma formation, lack of division of centromeres, first division or second division—can be eliminated naturally or experimentally, but unlike the mitotic cell the meiotic cell can neither be induced to revert back to a premeiotic state, nor can it be deterred from completing its destined role, however aberrant the products of meiosis might be. It would appear, at least at first sight, that the increased duration of the S period and the threefold increase in nuclear volume (Beasley, 1938) in meiotic cells, are reflections of this differentiated state, not necessarily the initiators of it.

One of the absolute and critical consequences of meiotic differentiation, however, is the separation of chromosome replication from cytokinesis. Indeed this may be the crucial differentiation, and has been suggested by the events taking place in *Neurospora* (Westergaard, 1964). In this fungus, meiosis immediately follows caryogamy. The time of chromosome reproduction is unknown. Synapsis occurs when the chromosomes are in a condensed state; the chromosomes uncoil to assume a typical pachytene appearance after which diplotene and the later events of meiosis follow in customary order. Two possibilities exist with regard to chromosome reproduction: Either DNA synthesis occurs at pachytene when chromosome length is at its maximum, or chromosome reproduction takes place prior to caryogamy, in which case DNA replication and crossing-over are unrelated events. It would appear that despite the cytological difficulties of *Neurospora,* this might well be a system in which the perplexing problems of synthesis and crossing-over might be profitably attacked.

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The Continuity of the Chloroplast in *Euglena*

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I. Introduction—the Two Aspects of Organelle Continuity

Eukaryotic cells contain several organelles conspicuously delimited from the surrounding cytoplasm at the level of resolution of the light microscope. This characteristic separates the vast bulk of living organisms from the prekaryotic group represented by blue-green algae and bacteria, and provides us with the problem of the origins of various organelles such as chloroplasts and mitochondria.

For convenience, two aspects of the problem can be distinguished. The first concerns the developmental origins of the organelle in question including a description of its formation from simpler precursor bodies together with a consideration of the control mechanisms governing the inception and orderly programming of the process. A second aspect concerns the continuity of the organelle from generation to generation and raises questions concerning the localization of information for the formation of organelle constituents and the mode of inheritance of this information during the reproductive cycle. Carried far enough back in time, this aspect includes speculations regarding the evolutionary origins of the organelle.

In discussing these questions, we will be primarily concerned with organelle continuity in *Euglena gracilis* var. *bacillaris* and with the chloroplast of this organism. Chloroplast development and inheritance in other organisms, particularly higher plants, has been reviewed extensively (Gibor and Granick, 1964; Granick, 1961, 1963; Rhoades, 1946; Cleland, 1962).

Much of the pioneering work on *Euglena* has been performed by Pringsheim, Lwoff, Hutner, Provasoli, and their collaborators. Through their efforts, the two strains of *Euglena* most popular for research were isolated and obtained in axenic culture; defined media were devised which

support luxuriant growth of the organism in a wide pH range (pH 3–9) on a variety of substrates. Their work also emphasized the suitability of *Euglena* for studies of chloroplast development by demonstrating that the formation of this organelle was light-dependent and that various agents would interfere with the ability of the cells to form plastids. This work is ably summarized in reviews (Hutner and Provasoli, 1951, 1955). The preceding applies only to two strains of *Euglena gracilis* since many other species of *Euglena* do not posses these characteristics. These two strains are *Euglena gracilis* var. *bacillaris* and *Euglena gracilis,* Z strain. Since the strains are very similar, *Euglena* will be used to designate them in the subsequent discussion whenever there is no reason to think that the findings reported are unique to one strain or the other. A useful *Euglena* bibliography will be found in Wolken (1961).

II. The Developmental Aspect of Chloroplast Continuity

A. *The Euglena Chloroplast*

The fully developed chloroplast characteristic of light-grown cells of *Euglena* consists of about 12 lamellae each composed of 2 to 4 closely appressed pairs of membranes called discs (Gibbs, 1960; Epstein and Schiff, 1961; Ben-Shaul *et al.,* 1964) as illustrated in Figures 1 and 4. As is characteristic of algal chloroplasts in general, there is a centrally located pyrenoid region which appears more dense in electron micrographs and in which the lamellae appear to be somewhat reduced in thickness. Plates of paramylum, the carbohydrate reserve of *Euglena* (a β-1:3 glucan) (Kreger and Meeuse, 1952; Clarke and Stone, 1960) flank the exterior of the pyrenoid and the entire plastid is surrounded by a double membrane. In the fluorescence microscope (where, under illumination with blue light, the chlorophyll emits a red fluorescence) most of the chlorophyll appears to be distributed in the lamellar regions with very little or no fluorescence within the pyrenoid region. Figure 1 also shows another algal

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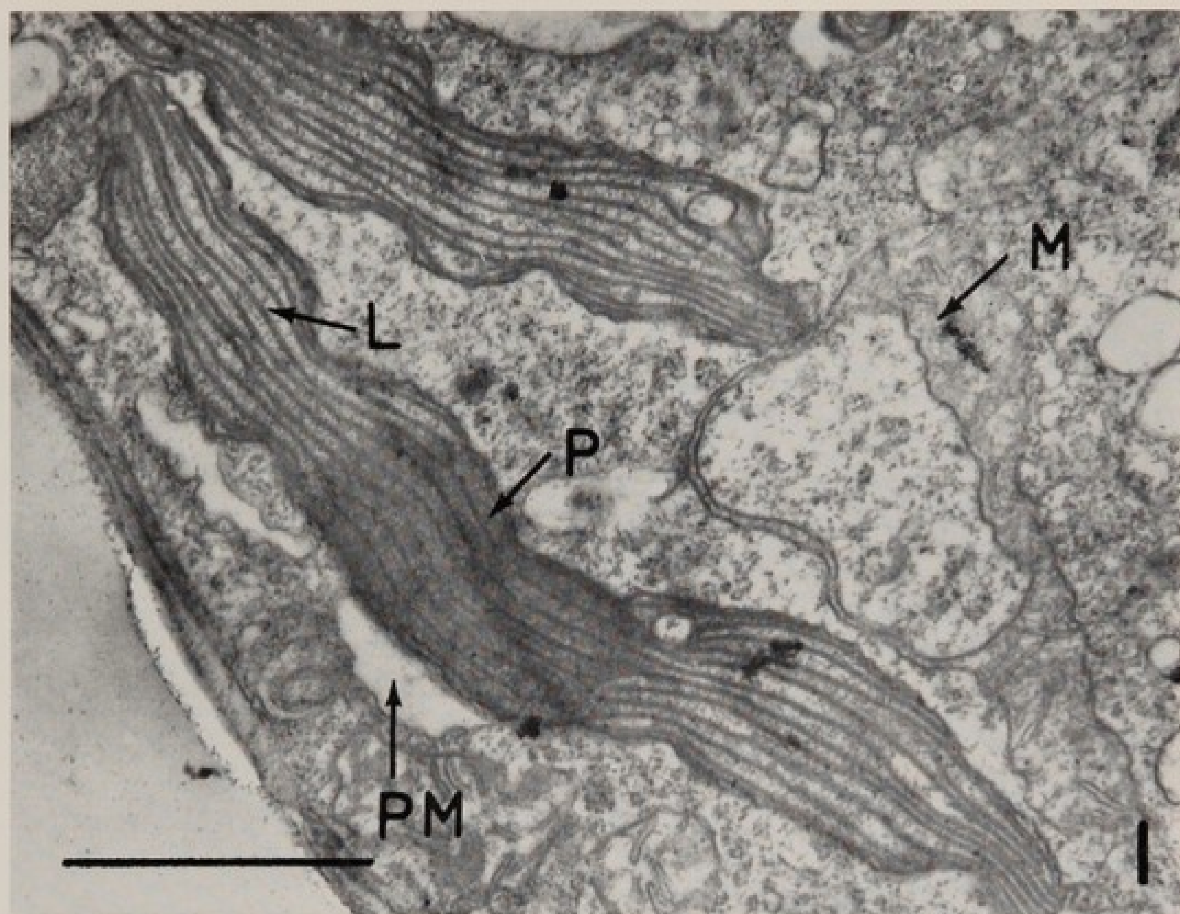
FIG. 1. Cell of wild-type *Euglena* grown in the light. (Ben-Shaul *et al.,* 1964.)

Abbreviations used here and in subsequent figures: B, basal body; C, chloroplast; D, disc; E, endosome; ER, endoplasmic reticulum; G, Golgi body; GU, gullet; L, lamella; M, mitochondrion; N, nucleus; NP, nuclear pore; P, pyrenoid; PE, pellicle; PM, paramylum; PP, proplastid; PV, pinocytotic vesicle. Marker indicates 1 μ. Here and in subsequent figures, the organism is *Euglena gracilis* var. *bacillaris.*

FIG. 2. Wild-type *Euglena* grown in the dark. (Ben-Shaul *et al.,* 1965.) (Key to abbreviations in legend of Fig. 1.)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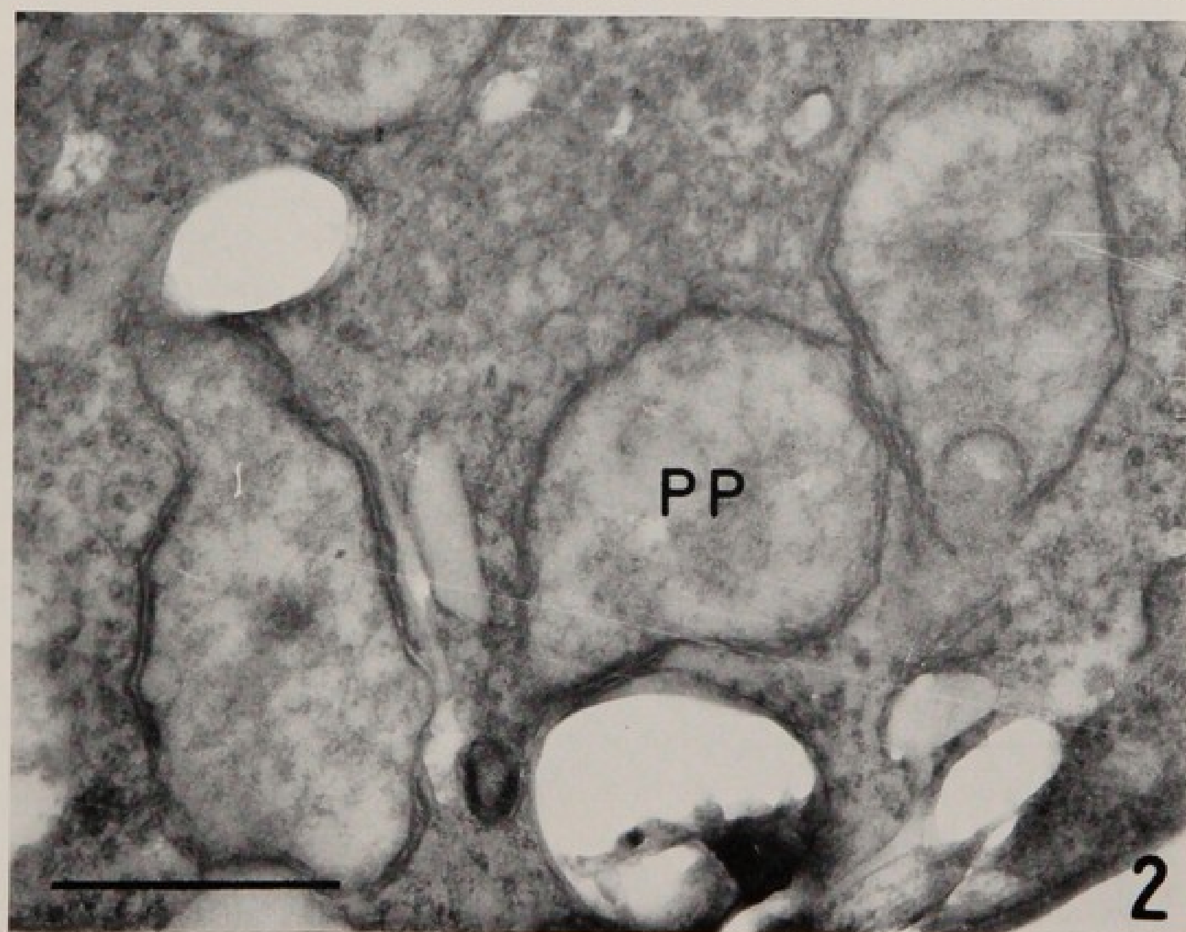
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characteristic shared by *Euglena,* the absence of grana regions in the chloroplast characteristic of higher plant chloroplasts.

B. *The Light Requirement for Chlorophyll and Chloroplast Formation*

We are accustomed to thinking of the formation of chlorophyll and chloroplasts in plants as a light-dependent process but in fact, many organisms form chlorophyll and chloroplasts in the dark. The angiosperms, in general, and *Euglena* and *Ochromonas* (Gibbs, 1962) require light for the formation of plastid structures and chlorophyll, but certain gymnosperms and several algae such as *Chlorella* and *Chlamydomonas* do not. Indeed it may be true that the majority of algae resemble *Chlorella* and *Chlamydomonas* in this respect although an experimental test of this requires that the organism in question be capable of organotrophic growth on a reduced carbon source in the dark. Organisms which cannot utilize organic substrates cannot be adequately tested.

Those organisms which require light for chloroplast development and chlorophyll formation all appear to utilize light energy for the conversion of protochlorophyll(ide) *a* to chlorophyll(ide) *a* and careful action spectra in higher plants match the absorption spectrum of protochlorophyll(ide). (In those cases where careful studies have been carried out protochlorophyllide has been shown to be the compound converted photochemically to chlorophyllide *a*. In *Euglena,* however, and several other organisms, it has not been determined whether protochlorophyll or protochlorophyllide is the photochemically reduced precursor. While it is likely that protochlorophyllide will also turn out to be the precursor in these cases, the designation "protochlorophyll(ide)" will be used to indicate the ambiguity.) This photochemical step has been studied in a holochrome particle extracted from beans, and much of the pioneering work was done in Smith's laboratory (J. H. C. Smith, 1958). While the action spectra measured for *Euglena* are consistent with those for higher plants, (Nishimura and Huzisige, 1959; Wolken *et al.,* 1955) more data are necessary to be certain. Due to the small amounts of protochlorophyll(ide) in dark-grown organisms, most action spectra have measured the effectiveness of various wavelengths in bringing about an accumulation of chloro-

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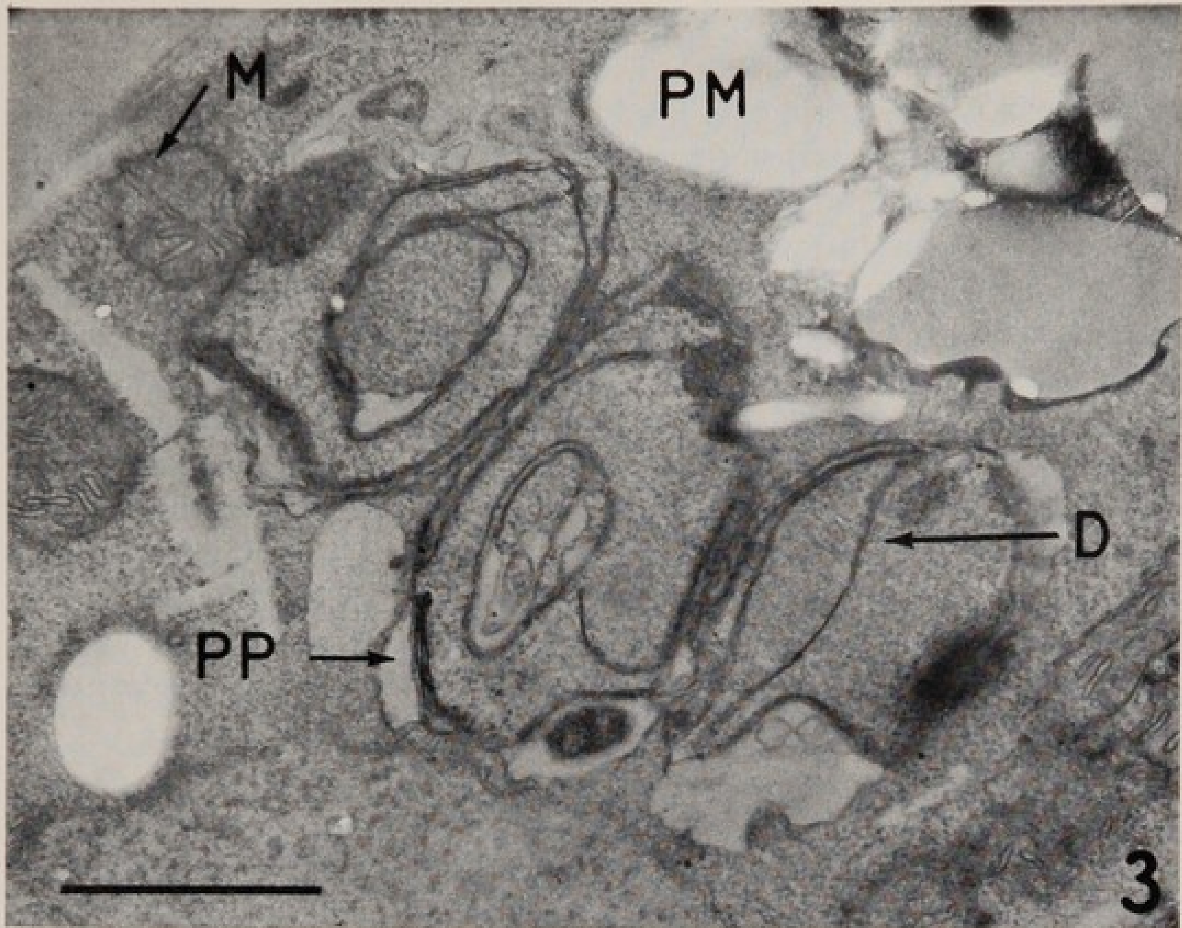
FIG. 3. Wild-type *Euglena* grown in the dark. (Liss *et al.,* 1965.) (Key to abbreviations in legend of Fig. 1.)

FIG. 4. Wild-type *Euglena* grown in the light. (Liss *et al.,* 1965.) (Key to abbreviations in legend of Fig. 1.)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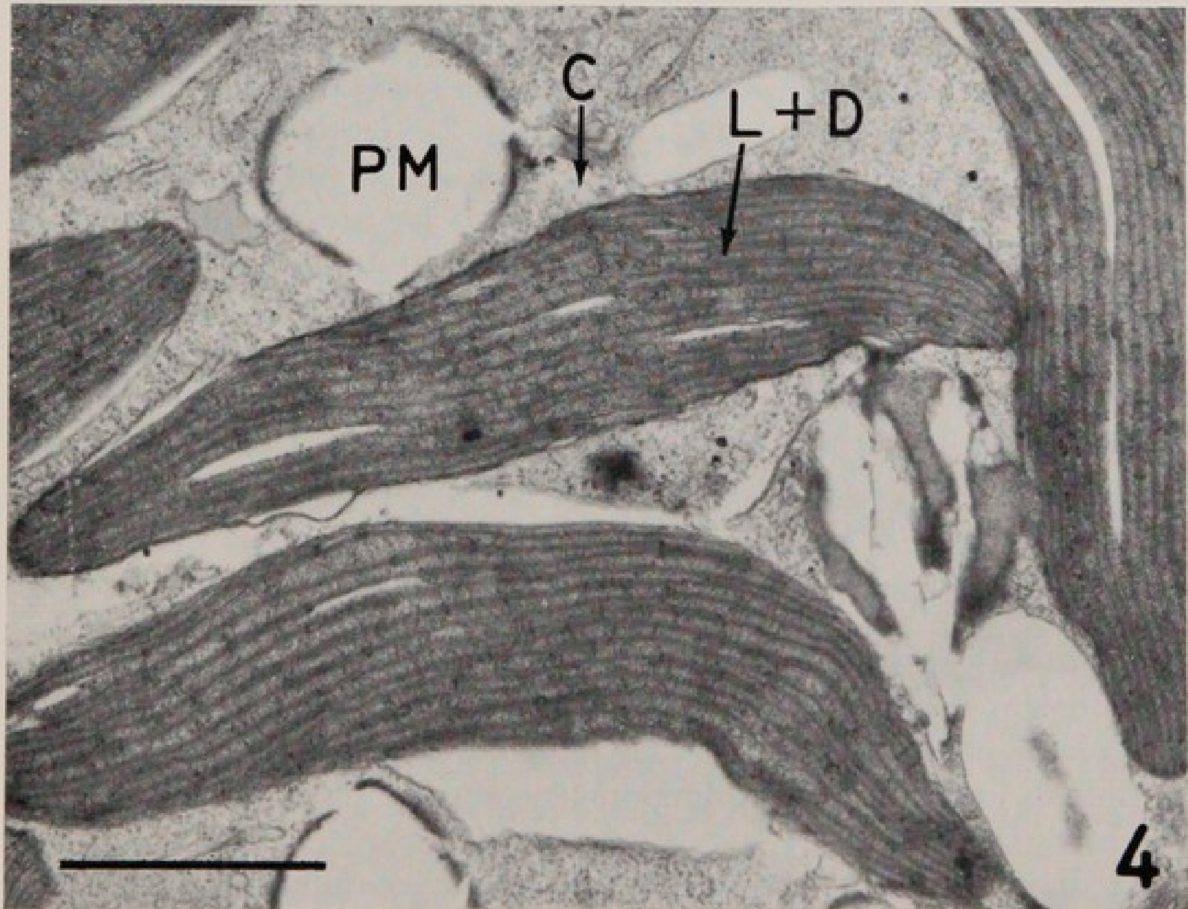
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phyll over a period of time. It would be very desirable, however, to have action spectra for the transformation of the initial protochlorophyllide to chlorophyllide since other photochemical transformations (possibly with different action spectra) may subsequently intervene.

From organisms which do not require light for chloroplast and chlorophyll formation, mutants can be isolated which require light. Thus, mutants of *Chlamydomonas* and *Chlorella* are known which are yellow when grown in the dark and become green only on exposure to light (Sager, 1958). If it is assumed that these mutants are the result of a single mutation in each case, it seems likely that the wild-type organisms contain both the standard light-induced protochlorophyll(ide) to chlorophyll(ide) step and an enzyme system for carrying out this transformation in the dark. Mutation would then have eliminated the dark enzyme leaving only the light-induced alternative, and rendering the mutant light-dependent for chloroplast and chlorophyll formation.

C. *The Proplastid in Dark-grown Euglena*

After many generations of growth in the dark, *Euglena* cells lose all of their chlorophyll and contain only protochlorophyll(ide) (Nishimura and Huzisige, 1959; Schiff *et al.*, 1961b) as the detectable green pigment. Early electron micrographs revealed that these cells contain proplastids about 1 μ in diameter which seem to lack extensive internal structure (Epstein and Schiff, 1961; Ben-Shaul *et al.*, 1964), (Fig. 2). Improvement of fixation procedures, however, has permitted the detection of one or two internal membranes in many of the proplastids which are occasionally organized for part of their length into a more complex structure (Fig. 3), and particles the size of ribosomes can also be seen (Liss *et al.*, 1965). Rough counts with the fluorescence microscope indicate that there are about 30 proplastids per cell (Epstein and Schiff, 1961; Epstein *et al.*, 1960). Extensive tubular structures, such as the prolamellar body found in certain proplastids of higher plants (von Wettstein, 1961) seem to be absent (Ben-Shaul *et al.*, 1964).

D. *Chloroplast Development in Euglena*

When dark-grown cells of *Euglena* are exposed to light, chloroplast development ensues whether the cells are dividing or not. By keeping the cells under nondividing conditions on a "resting" medium it has been possible to study chloroplast development uncomplicated by cell division (Stern *et al.*, 1964a).

The morphological events of this process may be summarized as follows

(Epstein and Schiff, 1961; Ben-Shaul *et al.*, 1964): (1) Membranes (which later form discs) are invaginated from the inner proplastid membrane; (2) These discs eventually fuse with each other along their length to form lamellae consisting of 2–4 discs; (3) The pyrenoid region differentiates after about 18–24 hours of development; (4) The maximum number of lamellae is formed by about 72 hours of development and chloroplast development is complete. The kinetics of this process are summarized in Fig. 5. The rise and fall in the number of discs reflects the initial formation of discs reaching a maximum number, cessation of disc formation, and fusion of discs to form lamellae. Lamella formation is linear from about 14 hours to maturity.

All parameters seem to increase by about a factor of 3 between 10 and 14 hours of development. This has been interpreted as a fusion of three proplastids to form one chloroplast at this point in development, which is consistent with some radiation data to be subsequently presented. The pattern of development in *Euglena* appears to be quite different from that in higher plants and other algae which have been studied (Gibbs, 1962; von Wettstein, 1961; Sager, 1958).

E. *Developmental Physiology of the Chloroplast in Euglena*

1. *Pigments.* After its initial formation on light induction of dark-grown cells (Nishimura and Huzisige, 1959; Schiff *et al.*, 1961b), chlorophyll *a* increases slowly up to about 10 hours (Stern *et al.*, 1964a). After 10 hours (Fig. 6) the rate of chlorophyll *a* formation increases dramatically and becomes essentially parallel to the rate at which lamellae are being formed. Chlorophyll *b* is difficult to measure because even in fully developed cells it constitutes only a small proportion of the total chlorophyll.

The dark-grown cell contains appreciable carotenoid (Stern *et al.*, 1964a; Krinsky and Goldsmith, 1960) although the proportion of the total cell content localized in the proplastid is not known. On light induction of chloroplast development the increase of carotenoids parallels that of chlorophyll *a* (Fig. 6) (Stern *et al.*, 1964a). The carotenoids of the dark-grown cell are: β-carotene, echinenone, euglenanone, cryptoxanthin, zeaxanthin, *trans*-antheraxanthin, *cis*-antheraxanthin, trollein, and hydroxyechinenone (Krinsky and Goldsmith, 1960). At about 4 hours of chloroplast development neoxanthin appears for the first time and continues to increase during development (Krinsky *et al.*, 1964). The fact that neoxanthin appears approximately at the inception point of photosynthetic competence is pregnant with speculative possibilities.

2. *Photosynthesis.* The first event which can be measured by gas

exchange is the large irreversible increase in the rate of oxygen uptake when dark-grown cells are exposed to light (Schiff, 1963). This undoubtedly represents an increase in respiration for mobilization of energy for the synthesis of chloroplast constituents. Superimposed on this large irreversible change is a small reversible photo-induced uptake of oxygen. As development of the chloroplast proceeds, this reversible uptake is

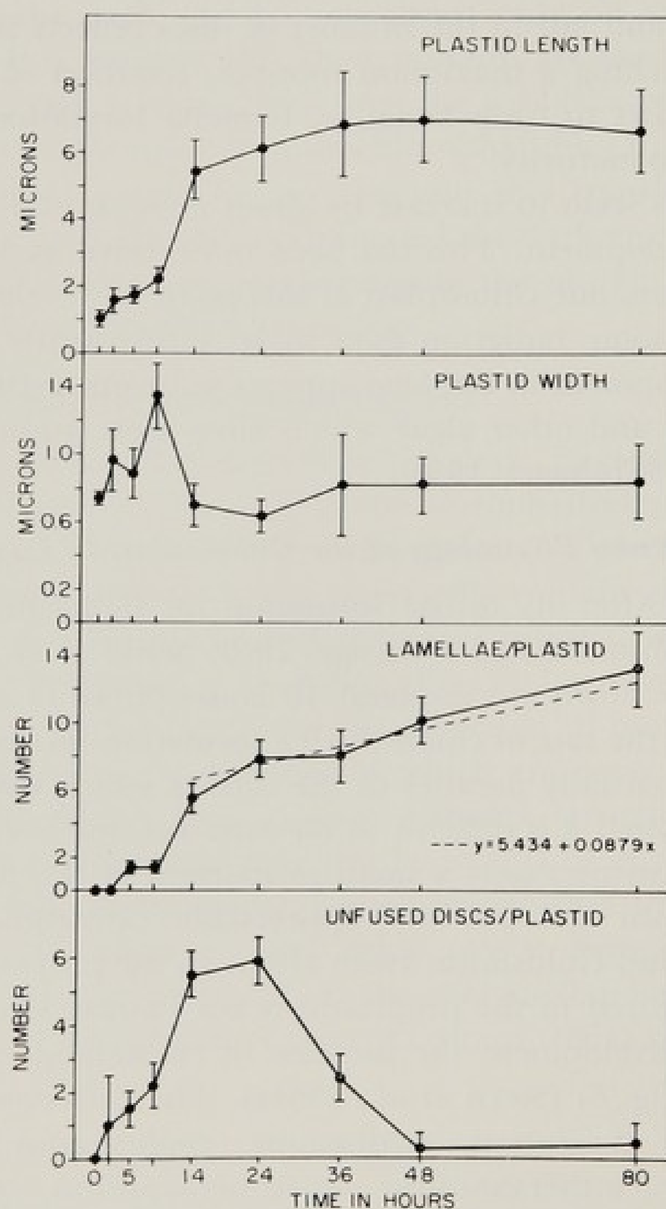


FIG. 5. Kinetics of the formation of discs and lamellae; and plastid lengths and widths during chloroplast development in *Euglena*. Zero time represents dark-grown cells immediately before induction of development with light. Time is measured from hours of development after dark-grown cells are exposed to light. In all cases, the points represent the means of several observations and the flags show the 95% confidence intervals of the means. The dotted line was fitted by least squares to the linear portion of lamella development. (Ben-Shaul *et al.*, 1964.)

gradually compensated until by about 4 hours of development it has become balanced by photo-induced oxygen evolution. From this point onwards there is an increasing net photosynthetic oxygen evolution (Fig. 6). The inception point for photosynthesis, therefore, can be assumed to be somewhat prior to 4 hours of development. Photosynthetic carbon dioxide fixation also becomes apparent at about 6 hours of development (Stern *et al.,* 1964a). By 15 hours (Fig. 6) of development the photosynthetic quotient is 1.0 and remains at this value during the remainder

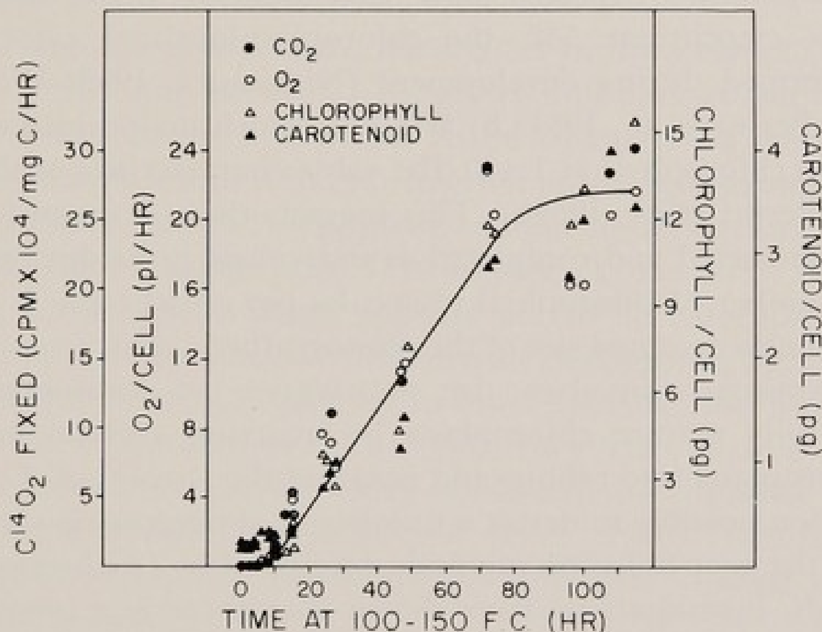


FIG. 6. Kinetics of the appearance of chlorophyll, carotenoids, photosynthetic oxygen evolution, and photosynthetic carbon dioxide fixation during chloroplast development in *Euglena*. As in Fig. 5 (data on comparable cells), zero time represents measurements on dark-grown cells; time is measured from inception of light-induced chloroplast development. (Stern *et al.,* 1964a.)

of development. In general, the rate of photosynthesis during development parallels the formation of pigments and lamellae (Stern *et al.,* 1964a) (Figs. 5 and 6). The optimum light intensity for development in *Euglena* is about 100 ft-c; higher or lower intensities yield lower amounts of chlorophyll. Low intensities also lead to abnormal develpment (Stern *et al.,* 1964b; Ben-Shaul *et al.,* 1964).

3. *Protein Synthesis.* Since chloroplast development takes place normally on a medium devoid of nitrogen and carbon sources, it is of interest to know whether the formation of the chloroplast involves *de novo* protein synthesis or, alternatively, whether the proteins of the chloroplasts already exist in the dark-grown cells and are merely packaged

into chloroplasts on light induction. Several enzyme activities have been shown to be associated with the chloroplast fraction of light-grown cells (Smillie, 1963).

One way of attacking the problem is to measure enzyme activities during development. TPN-Triose phosphate dehydrogenase (Fuller and Gibbs, 1959; Brawerman and Konigsberg, 1960), TPN-transhydrogenase (Lazzarini and Woodruff, 1964; Lazzarini and San Pietro, 1963), and ribulose diphosphate carboxylase (Fuller and Gibbs, 1959) all appear during development. By spectroscopic determination, it has been possible to show that cytochrome 552, the chloroplast-localized cytochrome of Euglena is formed during development (Nishimura, 1959; Wolken and Gross, 1963; Perini *et al.*, 1964a,b) at a rate which maintains the ratio of cytochrome to chlorophyll at 1:400, the value characteristic of the mature chloroplast (Perini *et al.*, 1964b). This suggests that photosynthetic units are being constructed and completed as individual units during development. The number of chlorophyll molecules per cytochrome is consistent with the currently accepted size of the photosynthetic unit.

Another approach involves the preparation of antibodies to the proteins of fully mature chloroplasts by injecting acetone powders of purified chloroplasts into rabbits and isolating the antiserum (Lewis *et al.*, 1965). It is then possible to detect a number of antigen-antibody reactions by means of the agar diffusion methods developed by Ouchterlony. Using these methods, investigations have shown that there is a progressive increase in the number of antigens in the cell which react with the anti-chloroplast antibodies as chloroplast development proceeds. These results suggest that new proteins are being formed during chloroplast development (Lewis *et al.*, 1965). Considered together, this evidence suggests that the induction of chloroplast development by light resembles a mass induction of adaptive enzymes. Many new proteins and enzyme activities appear during development and since this occurs on a medium lacking carbon and nitrogen, these molecules must be synthesized either from internal pools of intermediates or from the breakdown of existing macromolecules.

4. *RNA and Ribosomes during Development.* Extensive work on this problem has been performed using the Z strain (Smillie and Krotkov, 1960). Previous findings had indicated that exposure of dark-grown organisms to light brings about synthesis of chloroplast proteins and an increase and change in cellular RNA (Brawerman *et al.*, 1962a; A. O. Pogo *et al.*, 1962). Further explorations showed that the chloroplasts have distinctive ribosomes (Brawerman, 1963) which can be distinguished from

their cytoplasmic counterparts on the basis of base composition and sedimentation characteristics. It was further shown that intact chloroplasts from *Euglena* can incorporate amino acids into protein (Eisenstadt and Brawerman, 1964a,b). Ribosomes isolated from both chloroplasts and cytoplasm could incorporate amino acids into protein when provided with necessary cofactors and intermediates (Eisenstadt and Brawerman, 1964a,b). The evidence suggests that both the cytoplasm and chloroplasts of *Euglena* have protein synthesizing systems of the usual type found in other systems.

5. *Miscellaneous Compounds.* Differences in lipid content and type of lipid have been found between light-grown and dark-grown cells (Rosenberg, 1963). α-Linolenic acid seems to be characteristic of the *Euglena* chloroplast since it is found in that fraction of the light-grown cells, but is absent or present only in a very small amount in dark-grown cells and in cells which cannot form plastid structures (Erwin and Bloch, 1962). Ergosterol, on the other hand, appears not to be chloroplast-associated since it is found in comparable amounts in dark-grown, light-grown, and mutant cells (Stern *et al.*, 1960). As in other systems, iron is necessary for chloroplast development and iron deficiency can impede chlorophyll formation (Price and Carell, 1964). Aminotriazole inhibits chlorophyll formation in *Euglena* (Aaronson and Scher, 1960). Utilizable carbon sources appear to repress chloroplast development to some extent (App and Jagendorf, 1963).

6. *Control Mechanisms.* Possible control mechanisms for chloroplast development will be considered in the last section of this paper.

F. *The Return of the Chloroplast to the Proplastid Condition*

This is a process most easily studied in unicellular organisms since in multicellular organisms the plastids exist in a highly determinate structure, the leaf. In *Euglena,* however, the chloroplast is capable of returning to the proplastid condition on dark adaptation (Ben-Shaul *et al.*, 1965). In dividing cells, lamellae dissociate into discs which are progressively lost from the plastids at a rate of about 0.3 per generation, somewhat less than the rate predicted from simple dilution of chloroplast constituents among daughter cells (0.5 per generation). Chlorophyll, however, is initially lost at a rate of about 0.5 per generation. This observation suggests that when the light is turned off, chlorophyll synthesis stops immediately (as required by the known light requirement for the protochlorophyll(ide) to chlorophyll(ide) step) but that some discs and lamellae

continue to be made at a low rate. This evidence leads to the interpretation that synthesis of the messenger RNA for the production of the constituents of these structures may be repressed in darkness, but that the messenger has a sufficiently long lifetime to persist for a generation or two to permit the synthesis of constituents at a diminishing rate as the available messenger becomes diluted among daughter cells. After about 144 hours of darkness (8 generations) the chloroplast has regressed all the way to the proplastid condition (Ben-Shaul *et al.*, 1965).

Under nondividing conditions a different situation prevails (Ben-Shaul *et al.*, 1965). There is virtually no loss of structure over the course of 144 hours even though about 38% of the chlorophyll has been lost completely and another 50% has been converted to pheophytin (see Greenblatt and Schiff, 1959; Brown, 1963). This suggests that chlorophyll per se is not a determinant of the lamellar structures, a conclusion also reached by other workers with different material (von Wettstein, 1961).

Thus, chloroplast development from the proplastid on light induction takes place in both dividing and nondividing cells in an identical manner. The reverse process, conversion of chloroplasts to proplastids in darkness, takes place in dividing cells but not in nondividing cells. An explanation of these differences can be sought in control mechanisms which adapt *Euglena* to its ecological situation. The organism is a facultative phototroph or organotroph growing equally well by photosynthetic fixation of carbon dioxide or at the expense of reduced organic compounds in the medium. The dark-grown organisms containing only proplastids can live and multiply if a reduced carbon source is available to them. If no carbon source is available they will cease to divide but will live for quite a while. It is of great adaptive advantage for the organism, under these conditions, to be able to form chloroplasts as soon as light is available and to adopt a phototrophic mode of existence. Even in the presence of organic compounds, when the organism can divide, photosynthesis might still be a more efficient method of energy utilization. Thus, the organism is capable of forming chloroplasts either under dividing or nondividing conditions. Consider now the organism with fully developed chloroplasts. If light becomes limited two alternatives are available. If the medium is devoid of reduced carbon sources and growth is impossible in darkness the organism maintains its plastids which would be advantageous at the first reappearance of light. Should the medium contain reduced sources of carbon the organism divides and rapidly loses the excess baggage of the chloroplast, by returning them to the proplastid condition, while it exists as an efficient organotroph. During the course of evolution control mechanisms

have apparently been selected in *Euglena* which efficiently adapt it to prevailing environmental conditions (Ben-Shaul *et al.*, 1965).

III. The Replicative Aspect of Chloroplast Continuity

A. *Definition of the Problem*

On the basis of available information from many organisms, it is possible to make some general models of chloroplast inheritance. Figure 7 shows three extremes for the purposes of discussion. In the first alternative information for the construction of a plastid resides in the nucleus which

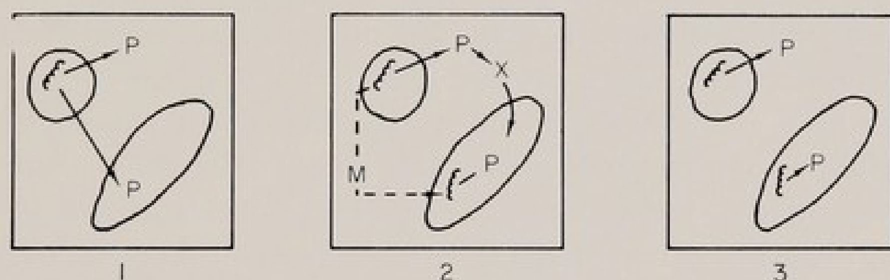


FIG. 7. Simplified hypothetical schemes for possible genetic interactions between organelles. In all cases, the circular structure represents the nucleus and the ellipse depicts the chloroplast. In alternative 1, an informational unit in the nucleus codes for the proteins of the entire cell including the chloroplast. Alternative 3 represents the other extreme in which the nucleus and chloroplast have independent informational units, the nuclear unit codes for generalized cell protein, and the chloroplast unit determines chloroplast proteins. Alternative 2 is necessitated by genetic studies with higher plants and shows two additional modes of interaction. The nucleus may code for a protein(s) which manufacture nutrients (X) required by the chloroplast. Alternatively, the nucleus may manufacture a mutagen (M) which irreversibly mutates the chloroplast informational unit.

codes for production of everything else in the cell as well. This model predicts that the chloroplast (or proplastid, or any other organelle) is constructed *de novo* in each generation from information supplied by the nucleus. The large numbers of mutations in maize, barley, and other organisms which affect chloroplast phenotypes and which behave in a perfectly Mendelian fashion can be used in support of this model. As we will see later, however, other interpretations of this data are possible.

The third alternative visualizes independent informational units in the nucleus and in the plastid. The unit in the nucleus codes for proteins produced in the nucleus and cytoplasm exclusive of the organelle in question. The organelle itself contains an informational unit which codes for its own proteins. When the cell replicates, the two informational units

are replicated independently leading to the possibility of autonomous chloroplast division.

In between these two extremes of interpretation are the possibilities for genetic interaction between the nucleus and the organelle shown in the second alternative of Fig. 7. These possibilities have been suggested by experiments with higher plants. Rhoades (1946) discovered a mutant affecting chloroplast phenotype in maize which he called *iojap*. The *iojap* gene is chromosomal and behaves in a Mendelian manner. Plants homozygous for the mutant gene produce abnormal chloroplasts. These abnormalities persist and are perpetuated, however, when the chloroplasts are crossed back into plants having a normal genetic constitution. Rhoades suggested, therefore, that the nucleus and plastid might have different genomes but that a mutant gene in the nucleus could produce a mutagen which irreversibly mutated the genome of the plastids which then continue to replicate the abnormality even after the nuclear constitution was returned to normal. Similar and even more complex interactions have been found in other plants, particularly *Oenothera* (Cleland, 1962).

Nuclear mutations affecting chloroplast phenotypes but which are entirely normal in Mendelian behavior (cited above in connection with alternative 1) can also be reinterpreted here. It is possible that the nucleus and plastid have independent genomes but that during the course of evolution the plastid has become nutritionally dependent on the rest of the cell for one or more metabolites (represented by "X" in alternative 2). A nuclear mutation which prevented the formation of these nutrients would lead to chloroplast abnormalities even though there was no direct informational dependence of the chloroplast on the nucleus.

In any case, the weight of the evidence seems to suggest separate genomes in nucleus and plastid. The rest of this paper will be concerned with evidence that the behavior of the *Euglena* system is consistent with this interpretation.

B. *Cytological Evidence for Chloroplast Division*

The fact that chloroplasts of algae divide has been known for many years and is well documented (Bold, 1951). In *Euglena deses,* for example, Gojdics was able to show that the cell divided first, apportioning the chloroplast complement approximately equally to the two daughter cells (Gojdics, 1934). After cell division was completed each chloroplast divided to restore the original plastid complement. An electron micrograph of a dividing chloroplast in *Euglena gracilis* var. *bacillaris* is shown in Fig. 8. Since the chloroplast returns to the proplastid condition in this organism,

and the number of proplastids remains approximately constant from generation to generation in the dark and since the dark-grown cells are always capable of chloroplast formation, it seems reasonable to assume that the proplastids in this species are also capable of division.

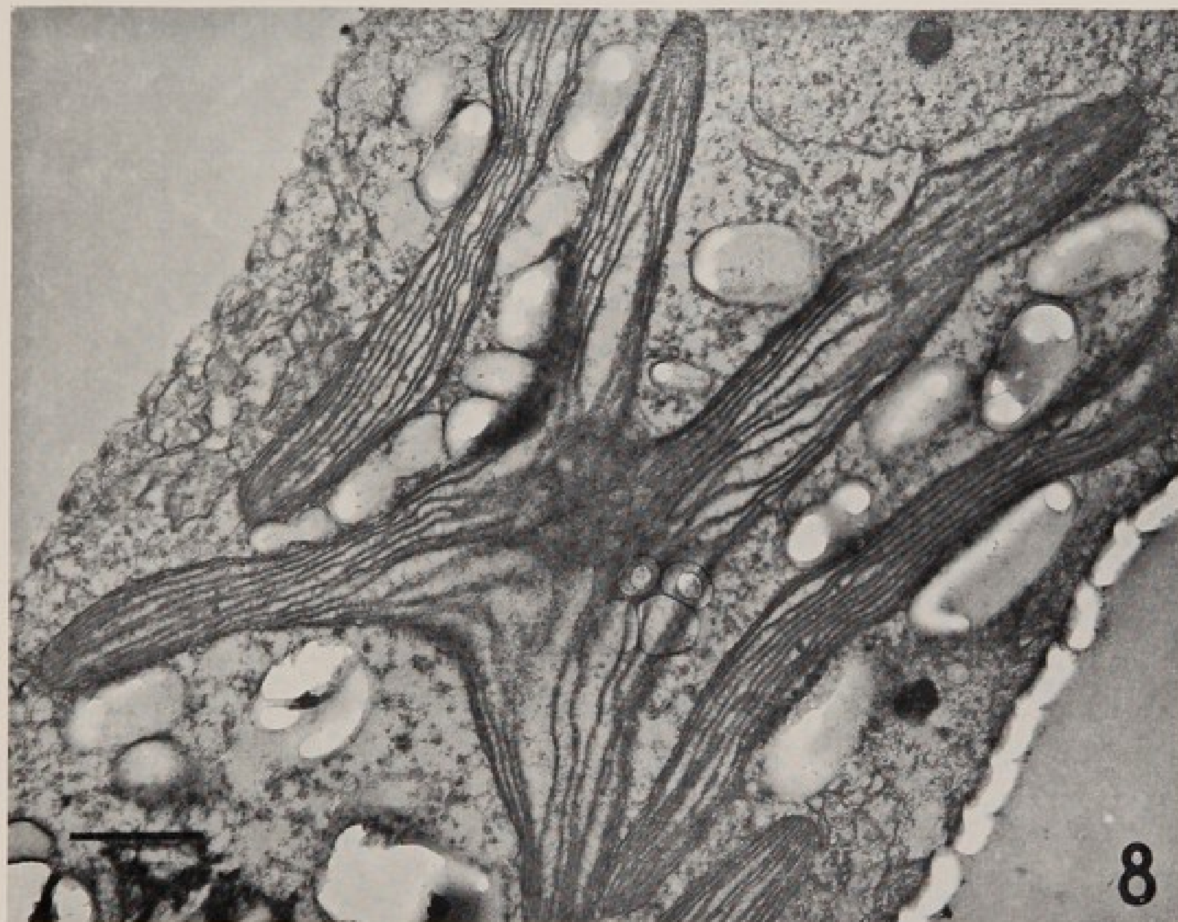


FIG. 8. Wild-type *Euglena* grown in the light in which a dividing chloroplast may be seen.

C. *Blockage of Green Colony-Forming Ability by Ultraviolet Light*

If light-grown or dark-grown cells of *Euglena gracilis* var. *bacillaris* are exposed to ultraviolet light and are plated under nonphotoreactivating conditions, but with light induction to permit chloroplast formation, the number of green colonies formed decreases as the ultraviolet dose is increased (Pringsheim, 1958; Lyman *et al.*, 1959, 1961). Increasing numbers of nongreen colonies replace the green colonies which are lost (Fig. 9). Thus, ultraviolet light brings about a loss of the ability to become green in the progeny of cells which had received the radiation. It is possible to bring about 100% conversion of the progeny to the nongreen condition at ultraviolet doses which are too low to have any effect whatsoever on

viability. Loss of viability begins at doses 10 times greater than the maximum doses required for inactivation of green colony-forming ability (compare Figs. 10 and 12) (Lyman *et al.*, 1961; Hill *et al.*, 1965a).

The first question which arose concerned the numbers of ultraviolet-sensitive entities controlling this process. Target analysis of the inactivation curves revealed that the curves for dark-grown and light-grown cells both fitted multiplicities of about 30 (Fig. 9) (Lyman *et al.*, 1961). This

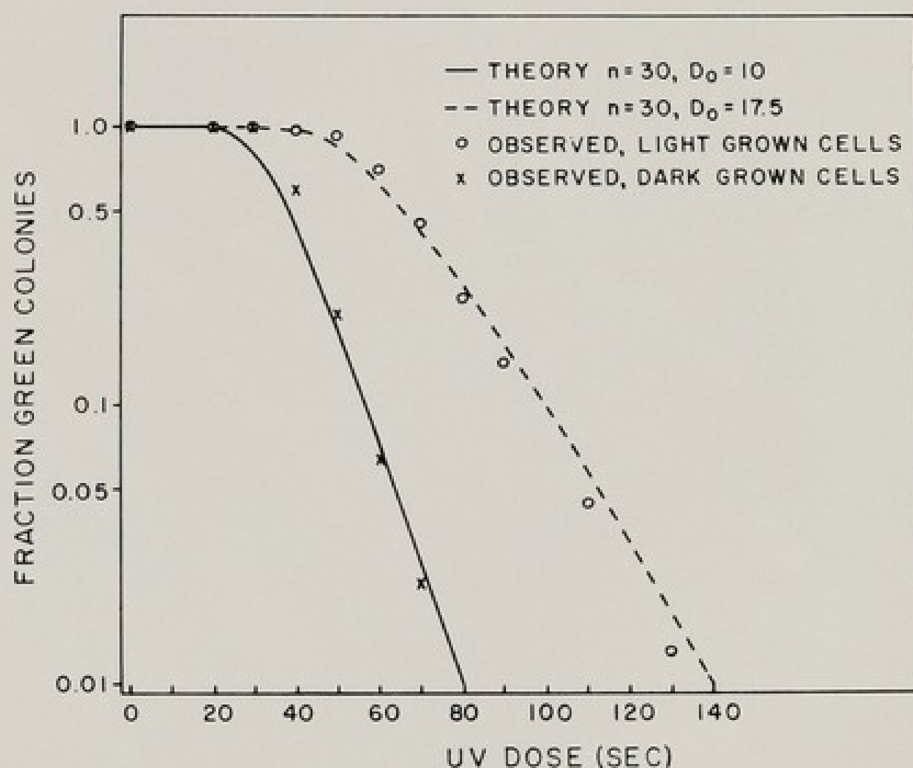


Fig. 9. Inactivation of green colony-forming ability in *Euglena* by ultraviolet light. The points represent experimental findings and the curves were calculated from target theory, multiplicity (n) of 30. D_0 is the dose required for a single inactivation. Light-grown and dark-grown cells show the same multiplicity but differ in D_0. (Lyman *et al.*, 1961.)

has since been verified by computer analysis of many experiments (Hill *et al.*, 1965a). This number agreed very nicely with the number of proplastids in the dark-grown cells estimated by fluorescence microscopy. The fact that light-grown cells having about 10 chloroplasts also showed ultraviolet multiplicities of 30 led us to the hypothesis that in the formation of 10 chloroplasts from 30 proplastids during light-induced development, proplastids fused in threes to form single chloroplasts conserving the ultraviolet-sensitive entities present in each proplastid. Evidence consistent with this interpretation was presented above in the discussion of

chloroplast development. On the basis of these facts and the lack of lethality in this ultraviolet range, we suggested that the ultraviolet-sensitive sites were cytoplasmic (Lyman *et al.*, 1961). Gibor and Granick (1962b) confirmed this by elegant experiments with an ultraviolet microbeam and were able to show that high doses of ultraviolet delivered selectively to the nucleus killed the cells, but low doses delivered to the surrounding cytoplasm, with the nucleus shielded, reproduced the phenomena we had described. It appears likely then, that the ultraviolet-sensi-

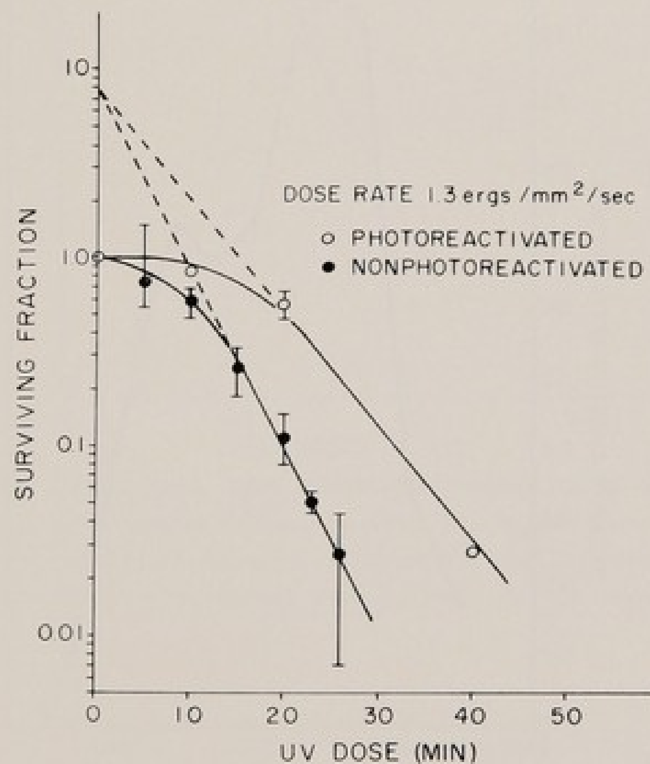


Fig. 10. Ultraviolet inactivation and photoreactivation of viability (colony-forming ability) in *Euglena*. The extrapolations indicate a multiplicity of about 8 for inactivation in this particular experiment. (Hill *et al.*, 1965a.)

tive entities affecting green colony-forming ability are localized in the plastids themselves.

The second question concerned the chemical nature of the ultraviolet-sensitive chromophore. An action spectrum for the ultraviolet inactivation revealed that the absorbing chromophore had peaks in the regions of 260 and 280 mμ suggesting the participation of a nucleoprotein (Fig. 11) (Lyman *et al.*, 1961).

As is true of ultraviolet inactivation in many other systems, the inactivation of green colony-forming ability in *Euglena* can be reversed by treatment with long wavelength ultraviolet and blue light if given soon after

inactivating ultraviolet (Schiff *et al.*, 1961a). This photoreactivation of green colony-forming ability can result in 100% reversal in *Euglena* (Fig. 12). This is a very high efficiency when compared with other systems (Jagger, 1958), and with the photoreactivation of viability in *Euglena* itself (Fig. 10) (Hill *et al.*, 1965a), and suggests that processes resembling multiplicity reactivation in bacteriophages may be occurring, resulting in cooperation among damaged entities in a single cell. This interpretation

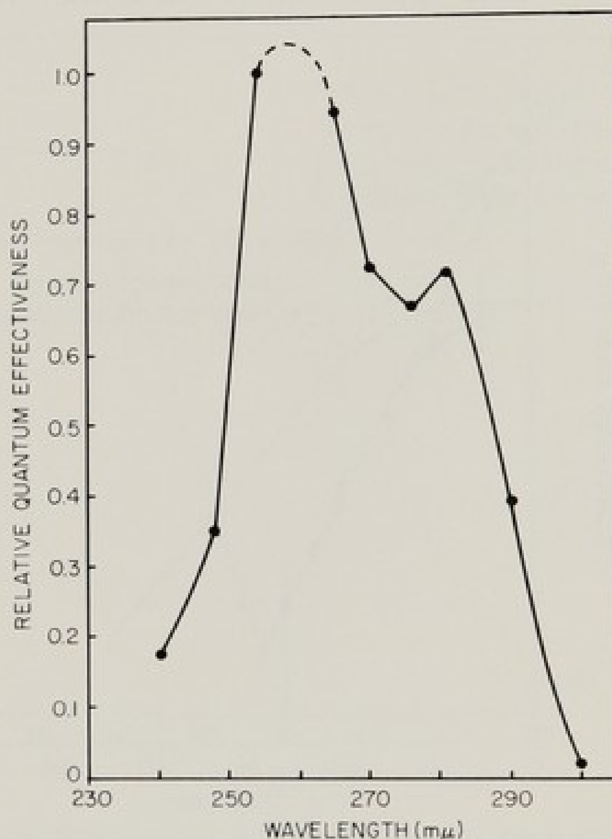


Fig. 11. Action spectrum for ultraviolet inactivation of green colony-forming ability in *Euglena*. (Lyman *et al.*, 1961.)

and others are discussed fully elsewhere (Hill *et al.*, 1965a,b,c). (For some anomalies in the Z strain, see Cook, 1963.)

Figure 10 also shows that multiplicities for inactivation of viability in *Euglena* are of the order of four to eight, consistent with other unpublished data from our laboratory using X rays. This suggests that the nuclear chromosomal complement in our strain of *Euglena gracilis* var. *bacillaris* is polyploid and helps to explain why the plastid system is so much more sensitive to ultraviolet than is viability.

The action spectrum for photoreactivation of green colony-forming ability in Euglena (Fig. 13) (Schiff *et al.*, 1961a) is very similar to that

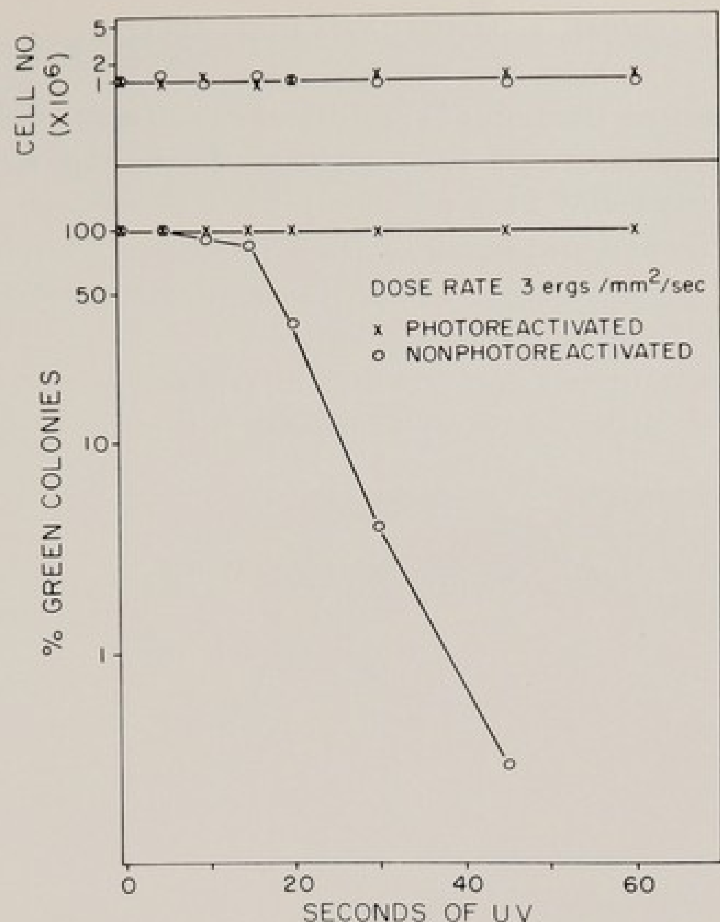


Fig. 12. Ultraviolet inactivation and photoreactivation of green colony-forming ability in light-grown cells of *Euglena* (lower curves). The upper part of the figure shows that the ultraviolet doses employed have no effect on cell viability as measured by viable cell numbers estimated from total colony count ("cell no." in upper part of figure). (Lyman *et al.*, 1961.)

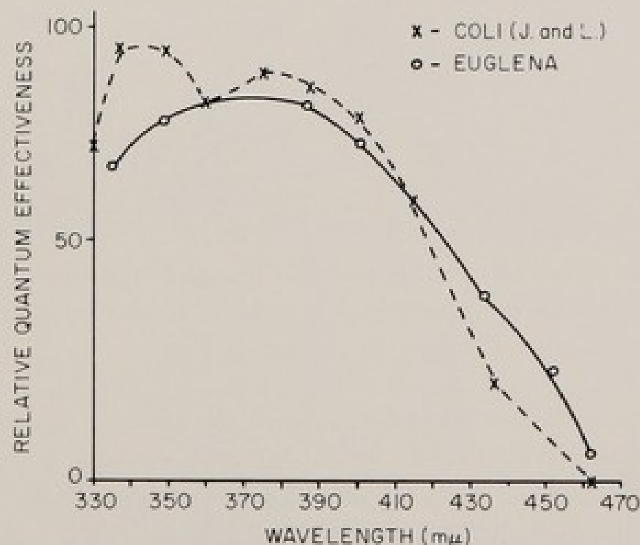


Fig. 13. Action spectrum for photoreactivation of green colony-forming ability in *Euglena* compared with photoreactivation in *Escherichia coli* measured by Jagger and Latarjet (1956). The rest of the visible spectrum (green to red) is completely inactive. (Schiff *et al.*, 1961a.)

described for photoreactivation of viability of *E. coli* and of photoreactivation of ultraviolet-inactivated bacteriophage T2 in the same organism (Kelner, 1951; Jagger and Latarjet, 1956). Since this system is known to involve the inactivation and repair of DNA it became possible to consider the possibility of plastid-localized DNA nucleoproteins which control the ability of the cells to produce green colonies.

It became important to know whether ultraviolet acted by preventing the replication of plastid entities or whether it merely prevented the

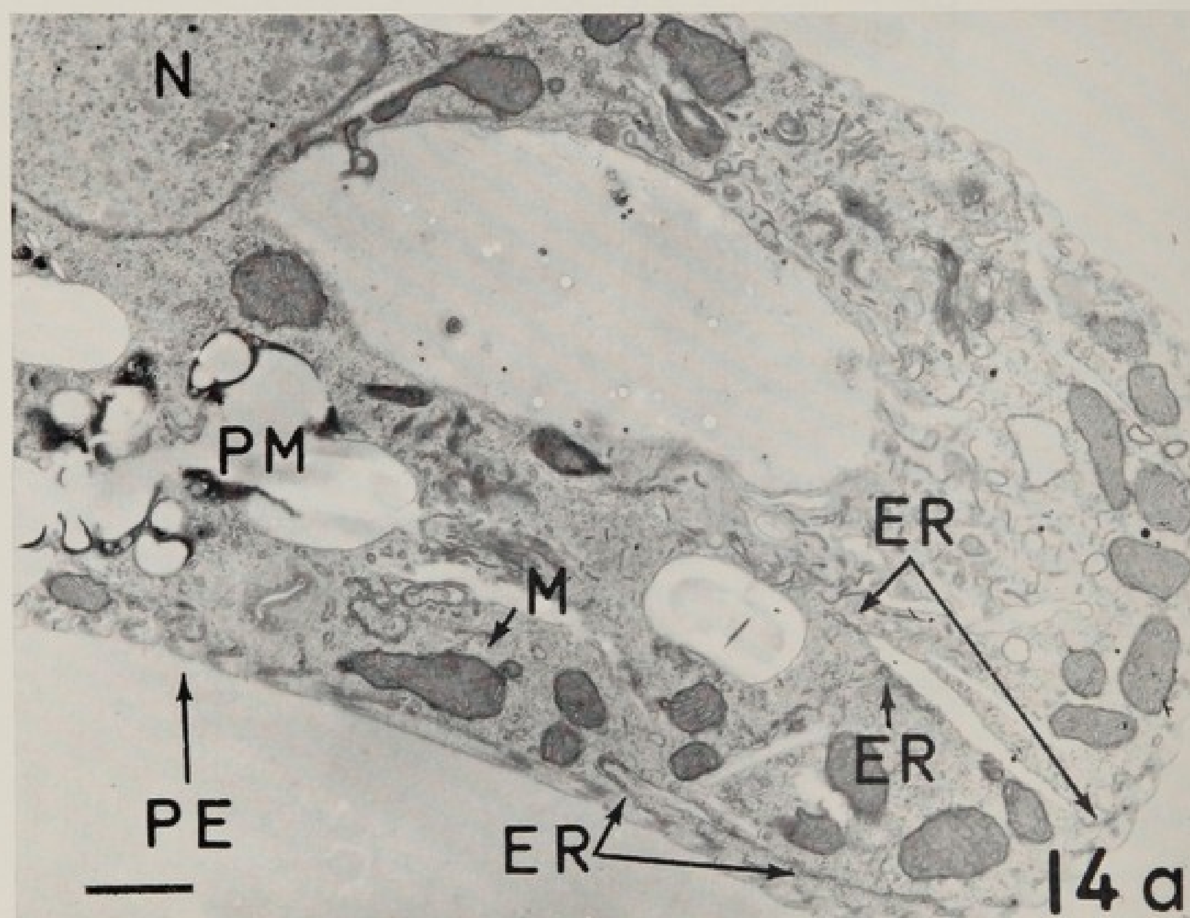
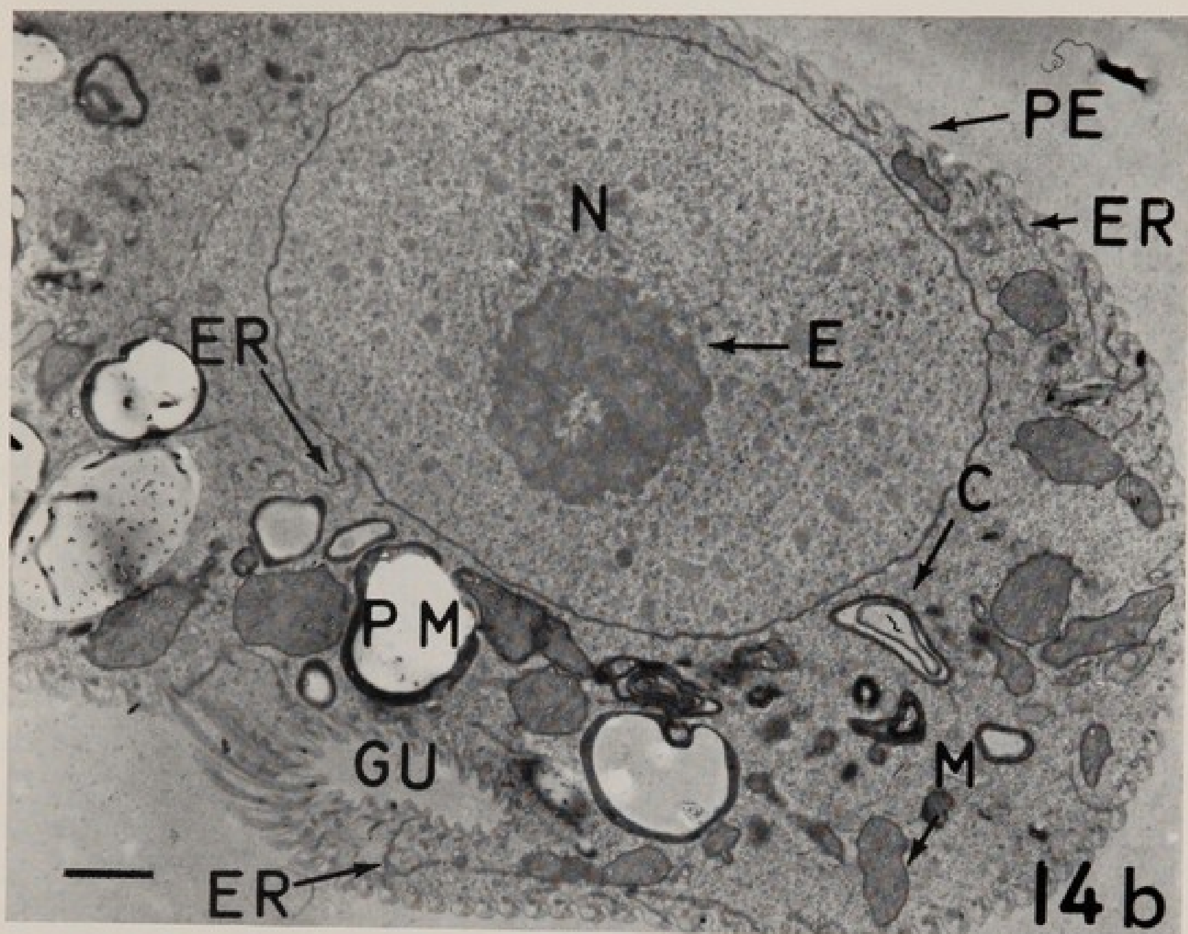


FIG. 14a. Y_3BUD, a yellow ultraviolet-induced mutant of *Euglena* grown in the dark. Note investment of pellicle by the endoplasmic reticulum and its continuation centrally. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

FIG. 14b. Y_3BUD, a yellow ultraviolet-induced mutant of *Euglena* grown in the light. Note connection of endoplasmic reticulum and nuclear membrane, and endoplasmic reticulum investment of pellicles of the exterior and of the gullet. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

FIG. 14c. W_3BUL, a white ultraviolet-induced mutant of *Euglena* grown in the light. Pinocytotic vesicles can be seen in association with the gullet. The questionable structures are believed (on the basis of many observations) to be either abnormal mitochondria or amyloplasts. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)


PE
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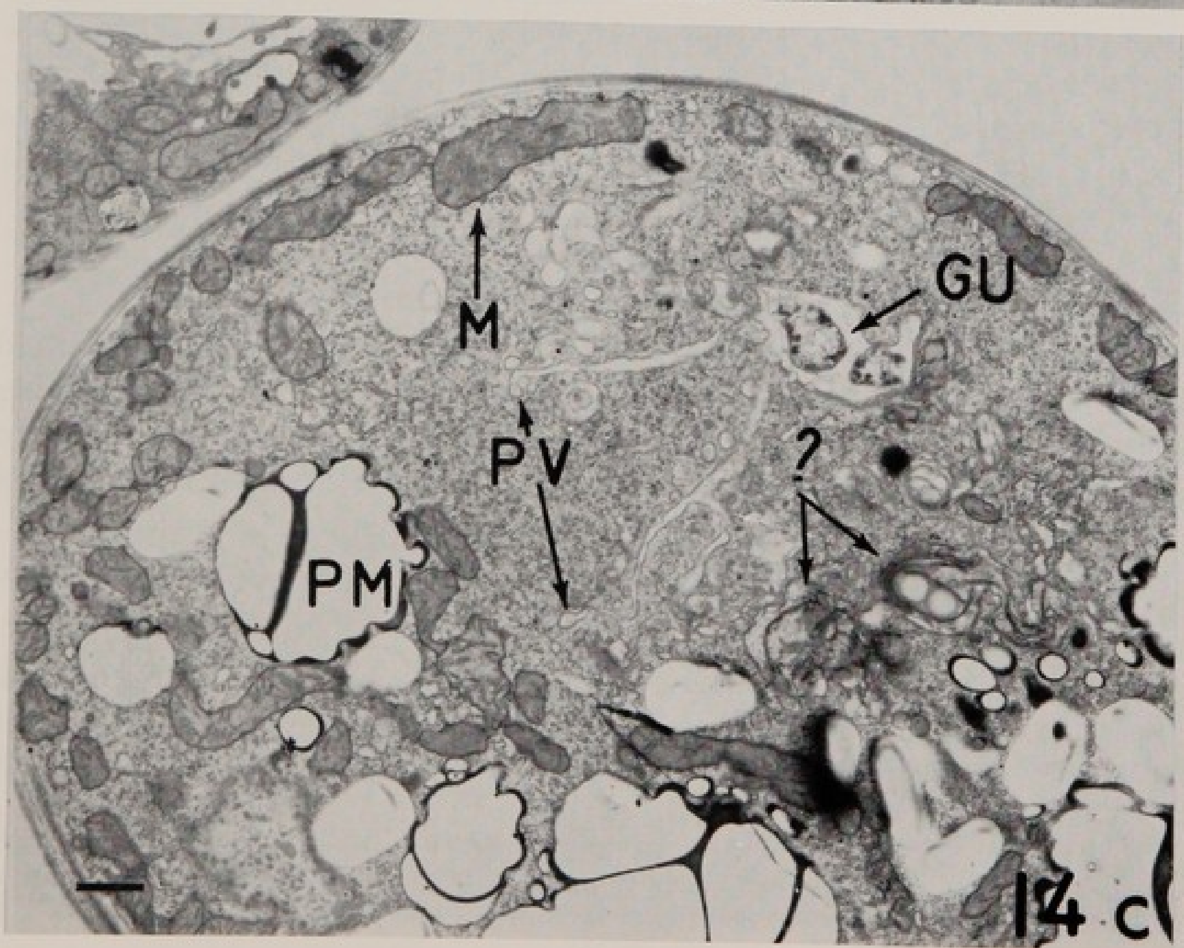
14 b


M
GU
PV
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14 c

development of proplastids into chloroplasts. An experiment was devised in which dark-grown cells were irradiated with a dose of ultraviolet which would produce 100% inactivation of green colony-forming ability when plated under nonphotoreactivating conditions. An aliquot of these cells was placed on a resting medium which prevented cell division and the cells were exposed to red light which induces chloroplast and chlorophyll formation without causing photoreactivation. Fluorescence microscopy revealed that all of the irradiated cells produced normal chlorophyll and chloroplasts indicating that chloroplast *development* was not blocked by ultraviolet. When the cells which had formed chloroplasts were plated however, they formed 100% nongreen colonies indicating that ultraviolet blocks the *replication* of chloroplast-forming entities at the time of cell division (Schiff *et al.*, 1961b).

The experiments with ultraviolet and photoreactivation lead to the assumption that the dark-grown cells contain about 30 DNA-protein entities localized in the approximately 30 proplastids. On light induction, the 30 proplastids develop into 10 chloroplasts by fusion in threes with the conservation of the 30 DNA-protein entities now localized 3 to a chloroplast. Irradiation of either the dark-grown or light-grown cells with ultraviolet results in blockage of either proplastid or chloroplast replication, respectively, at the time of cell division. This would predict then, that ultraviolet inactivation and loss of green colony-forming ability by a cell should be synonymous with the loss of that cell's plastids and their contained DNA entities.

D. *Cytology of Ultraviolet Mutant Cells*

Cells incapable of chloroplast and chlorophyll production produced through ultraviolet treatment have never reverted to chloroplast-forming competence in the 8 years these cells have been carried in culture. In addition, fluorescence microscopy reveals no red-fluorescing structures of any kind indicating the absence of protochlorophyll(ide) as well.

To confirm the absence of plastid structures, an extensive program of

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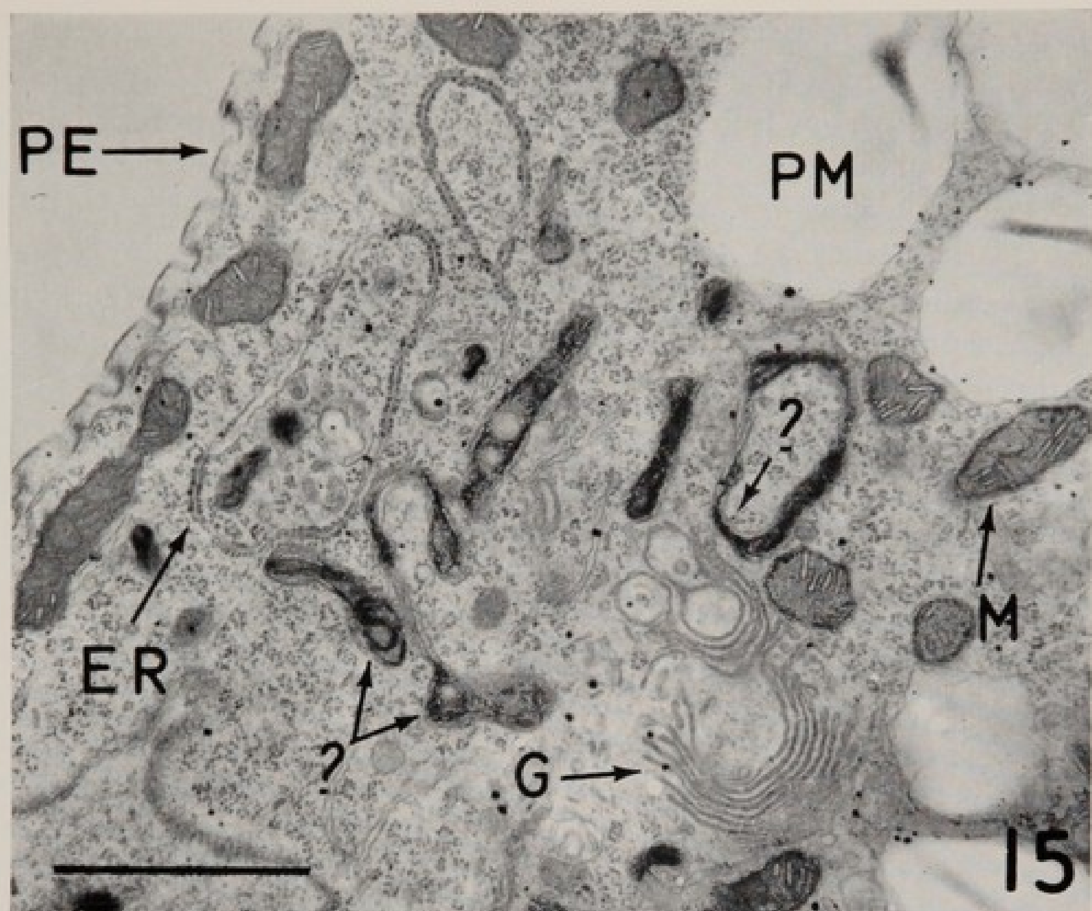
FIG. 15. W_3BUL, a white ultraviolet-induced mutant of *Euglena* grown in the dark. Structures marked with question marks are unknown but do not appear (on the basis of many observations) to be proplastids. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

FIG. 16. W_3BUL, a white ultraviolet-induced mutant of *Euglena* grown in the light. Note diversity of mitochondrial morphology, nuclear pores, and the absence of plastid-related structures. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)


PE
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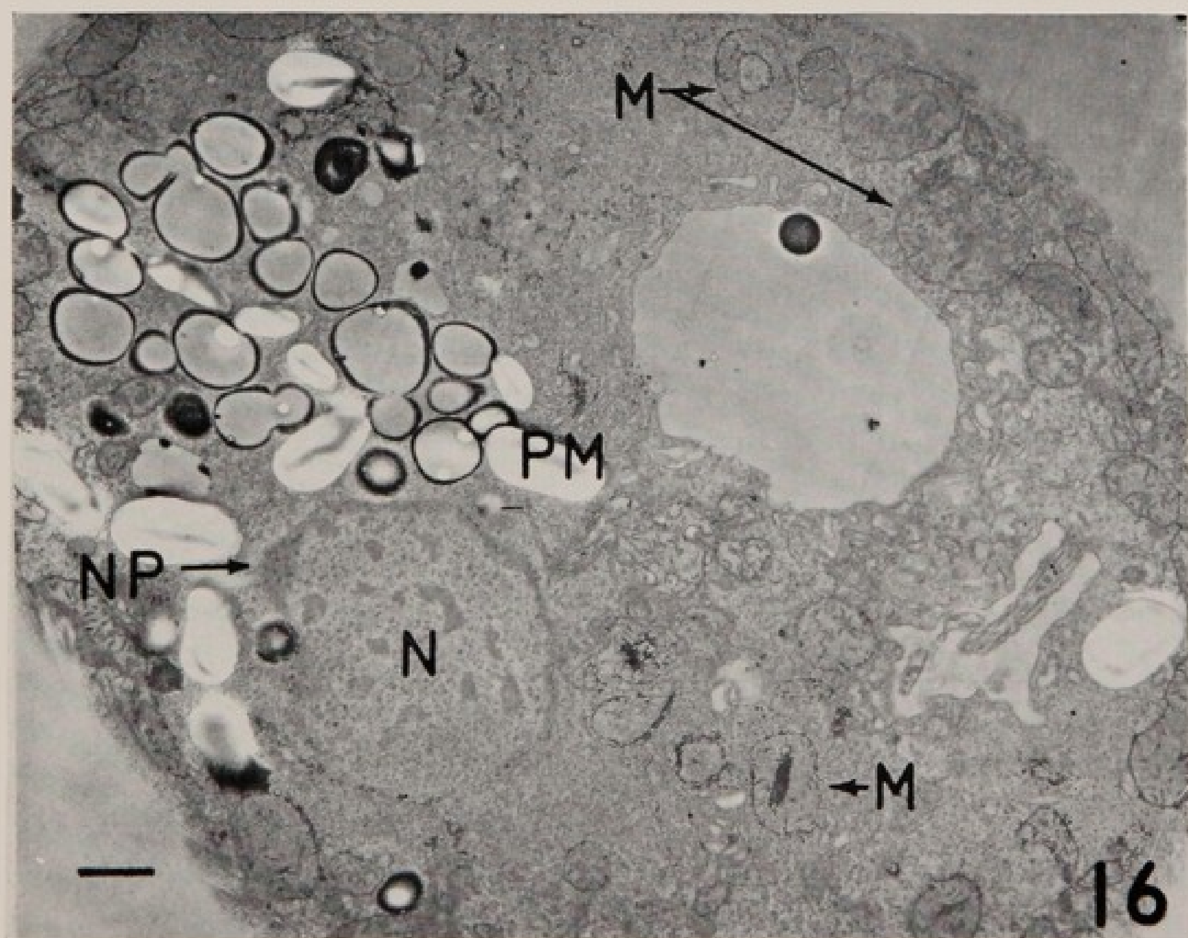
15


M
PM
NP
N
M
16

electron microscopy of *Euglena* mutants was undertaken. Improvement of fixation and embedding procedures (Liss *et al.*, 1965) now results in an image of proplastids and chloroplasts which stand out clearly and show enough individuality not to be confused with other organelles (Figs. 3 and 4). Other structures improve in resolution as well, including the endoplasmic reticulum (Fig. 14) (Liss *et al.*, 1965). Using these new methods, we have carefully examined the light-grown cells and dark-grown cells of all of our mutants and find that in the case of chlorophyll-free mutants induced by ultraviolet, streptomycin, and heat, there are no detectable chloroplasts or proplastids (Liss *et al.*, 1965). Representative pictures are shown in Figs. 15–20. Gibor and Granick (1962a) have treated mutants of this type from the Z strain with δ-aminolevulinic acid by freeze thawing the cells in a solution of the compound. They report that red-fluorescing centers can be detected in the cells after this treatment and interpret this to mean that plastids, or remnants of their structure, persist in the mutants. (See also Siegesmund *et al.*, 1962, and Moriber *et al.*, 1963.) No emission spectra are reported, however, for these red-fluorescing centers and it remains possible that they are mitochondrial sites of protoporphyrin synthesis for heme and cytochrome production rather than centers which form magnesium-containing tetrapyrroles, since both classes of compounds fluoresce red.

On the basis of our own work cited above and to be detailed below, we consider it unlikely that plastid or proplastid structures persist in clones of certain mutants of *bacillaris* induced with ultraviolet, heat, or streptomycin.

In addition to these mutants induced by ultraviolet, streptomycin, or heat which lack the ability to form any plastid structures or chlorophyll, others have been isolated which are blocked at some stage of development. All of these have proplastids in the dark (like wild type, Fig. 4) but display interesting abnormalities which should be of considerable use in studying the physiological basis of development (Liss *et al.*, 1965). For example, pale-green mutants (P_1) have been isolated with lack patches of lamellae and make less chlorophyll than wild type (Figs. 21 and 22) (Stern *et al.*,

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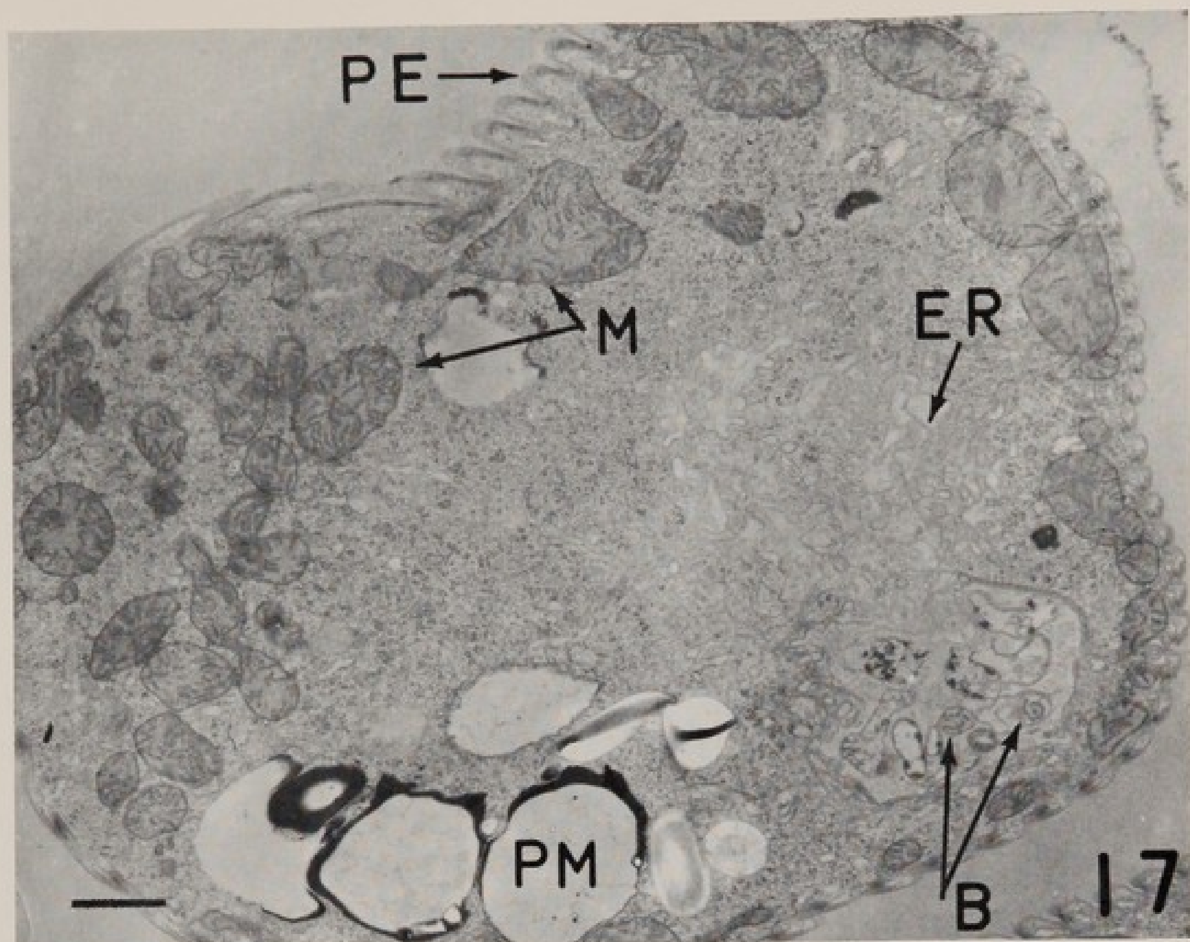
FIG. 17. $W_{10}BS_mL$, a white streptomycin-induced mutant of *Euglena* grown in the dark. Note absence of plastid-related structures and the extensive smooth endoplasmic reticulum. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

FIG. 18. $W_{10}BS_mL$, a white streptomycin-induced mutant of *Euglena* grown in the light. Note absence of plastid-related structures. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)


PE
M
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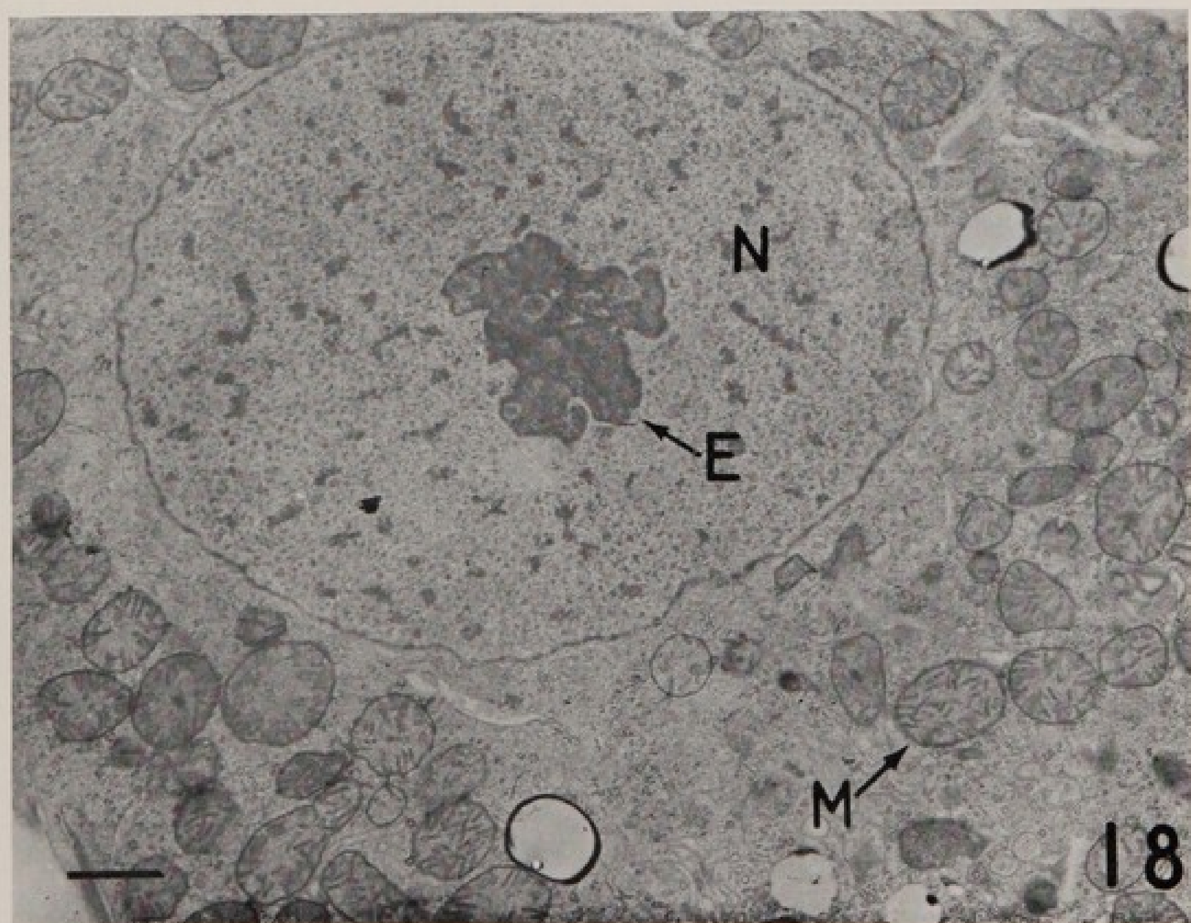
B
17


N
E
M
18

1964a). Olive-green mutants have abnormally large plastids with discontinuous lamellae and many unfused discs as in O_1 (Figs. 23 and 24). Another olive-green mutant, O_2 (Figs. 25 and 26) produces structures strangely reminiscent of the grana of higher plant chloroplasts. Yellow mutants have been obtained which are blocked rather early in development and form only small abnormal plastids. Y_1 and Y_3 (Figs. 27–30) are examples (Stern *et al.*, 1964a).

The mutant cells also display a phenomenon noted for dark-grown cells (Lefort, 1964); there is a great hypertrophy of mitochondrial structures of bewildering diversity. It is possible that the presence or absence of an active chloroplast controls the development of mitochondrial structures for compensatory respiratory activity.

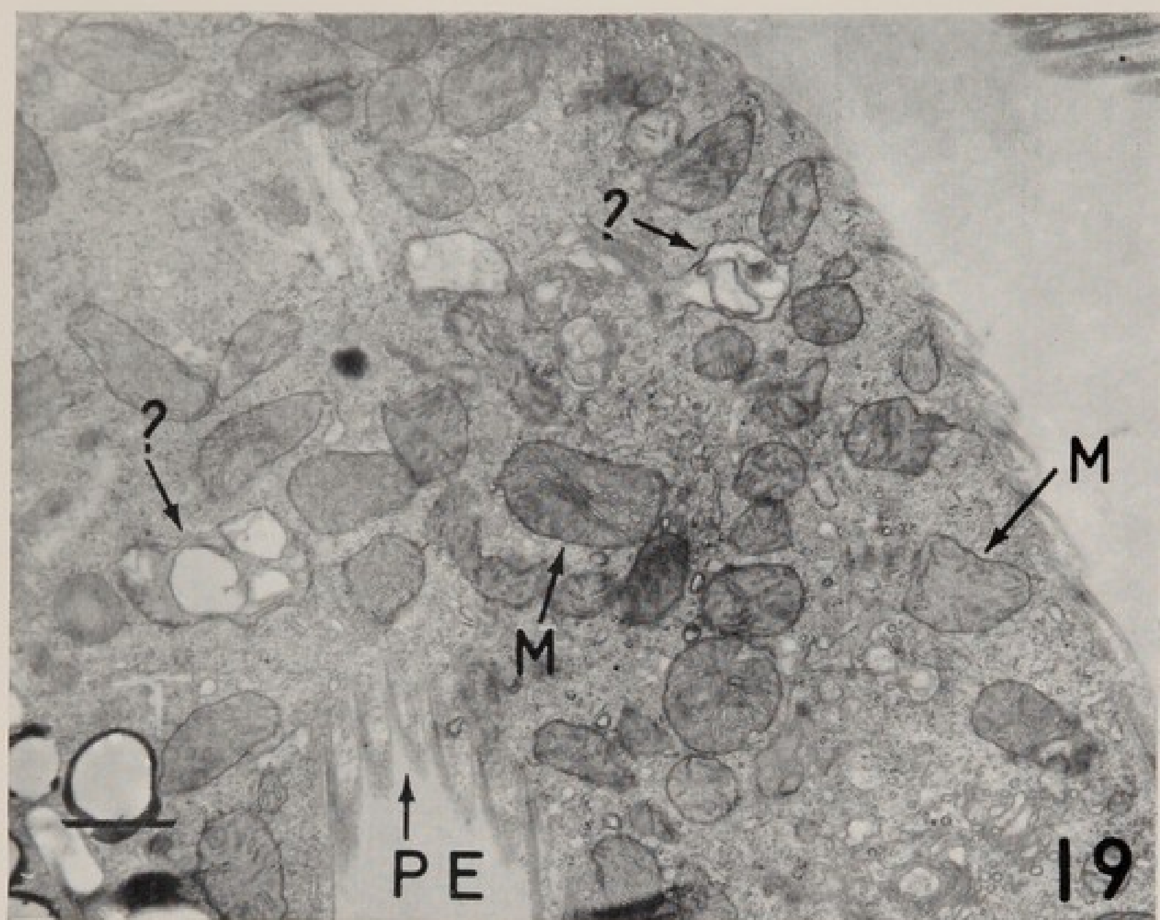
It is also possible that the mutagenic agent used in each of the cases to produce mutants in chloroplast phenotype also produced mutations in some of the mitochondria yielding some abnormal forms. Evidence for mitochondrial DNA will be discussed below.

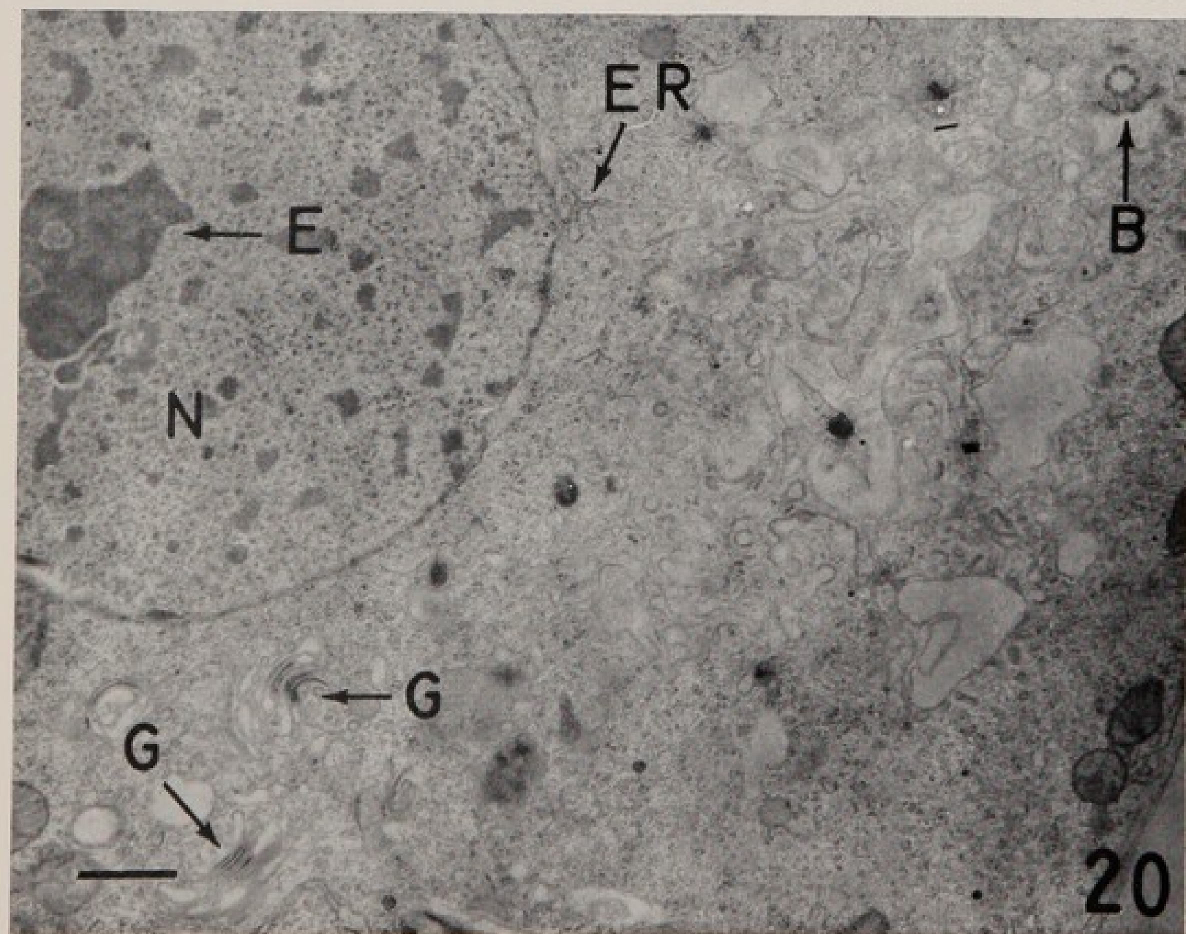
E. *DNA of the Chloroplast and Other Organelles*

The experiments with ultraviolet light described above suggested very strongly that a plastid-localized species of DNA exists in *Euglena*. Evidence in support of this was forthcoming from a comparison of the banding profiles of *Euglena* DNA in cesium chloride density gradients (Leff *et al.*, 1963). As may be seen in Fig. 31, light-grown cells display two bands of different densities, one at 1.708 and a satellite band at 1.688. Both of these are double-stranded since heat denaturation brings about the expected shifts to higher densities. Corresponding DNA preparations from ultraviolet mutants (Fig. 31) which lack plastid structures entirely, retained the main band at density 1.708 but showed no detectable satellite DNA. This suggested that the main band (1.708) is probably nuclear DNA while the satellite (1.688) is probably chloroplast-associated DNA. A similar situation was found in a comparison of *Chlamydomonas* DNA with its aplastidic counterpart, *Polytoma* (Fig. 31) (Leff *et al.*, 1963).

FIG. 19. W_8BHL, a white heat-induced mutant of *Euglena* grown in the dark. On the basis of many observations, the structures with question marks do not resemble proplastids; they may be amyloplasts. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

FIG. 20. W_8BHL, a white heat-induced mutant of *Euglena* grown in the light. Note absence of plastid-related structures and presence of basal body, connection of endoplasmic reticulum and nuclear envelope, and Golgi lamellae with dense intercisternal elements. (Liss *et al.*, 1965.) (Key to abbreviations appears in legend of Fig. 1.)


?
?
M
M
PE
19


ER
B
E
N
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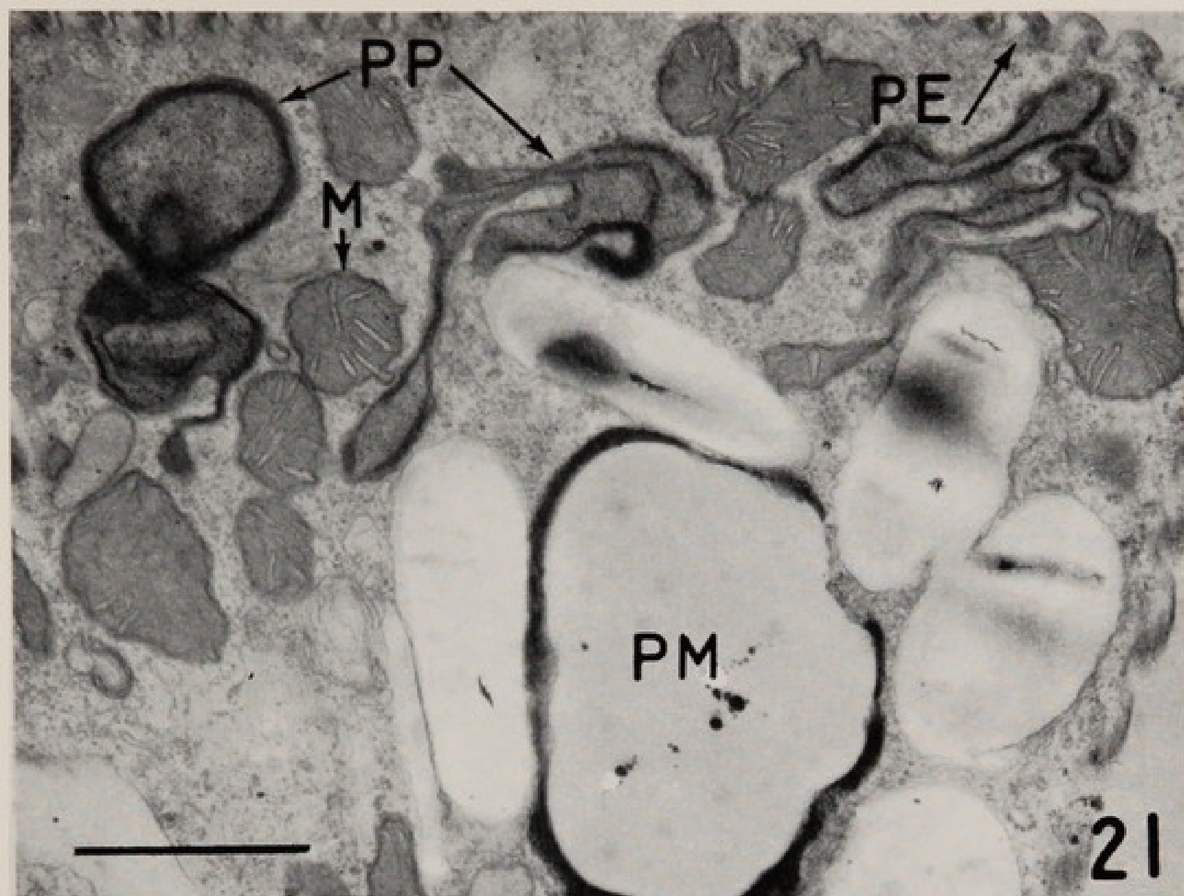
G
20

FIG. 21. Proplastids of P_1BXL, a pale-green X ray-induced mutant of *Euglena* grown in the dark. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

FIG. 22. Plastids of P_1BXL, a pale-green X ray-induced mutant of *Euglena* grown in the light. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)


PP
PE
M
PM
21

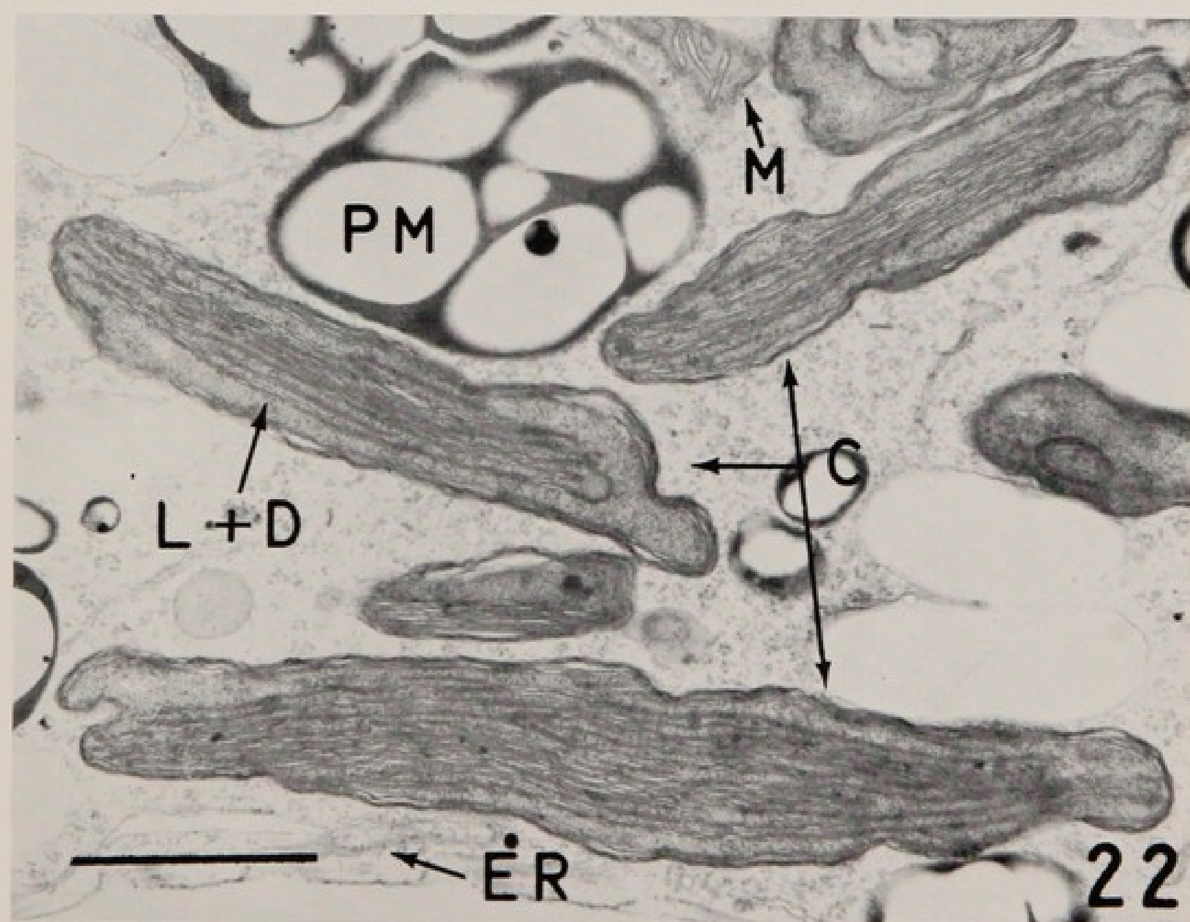
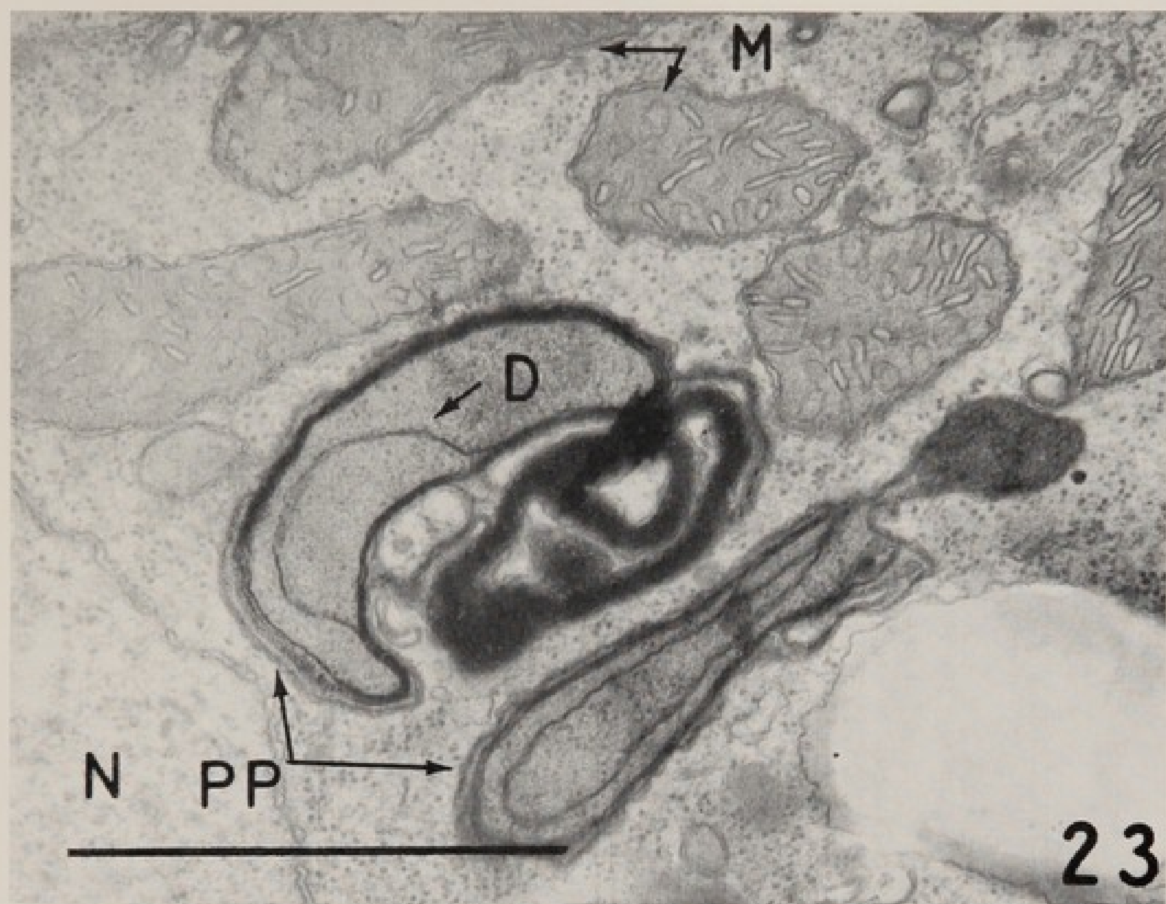
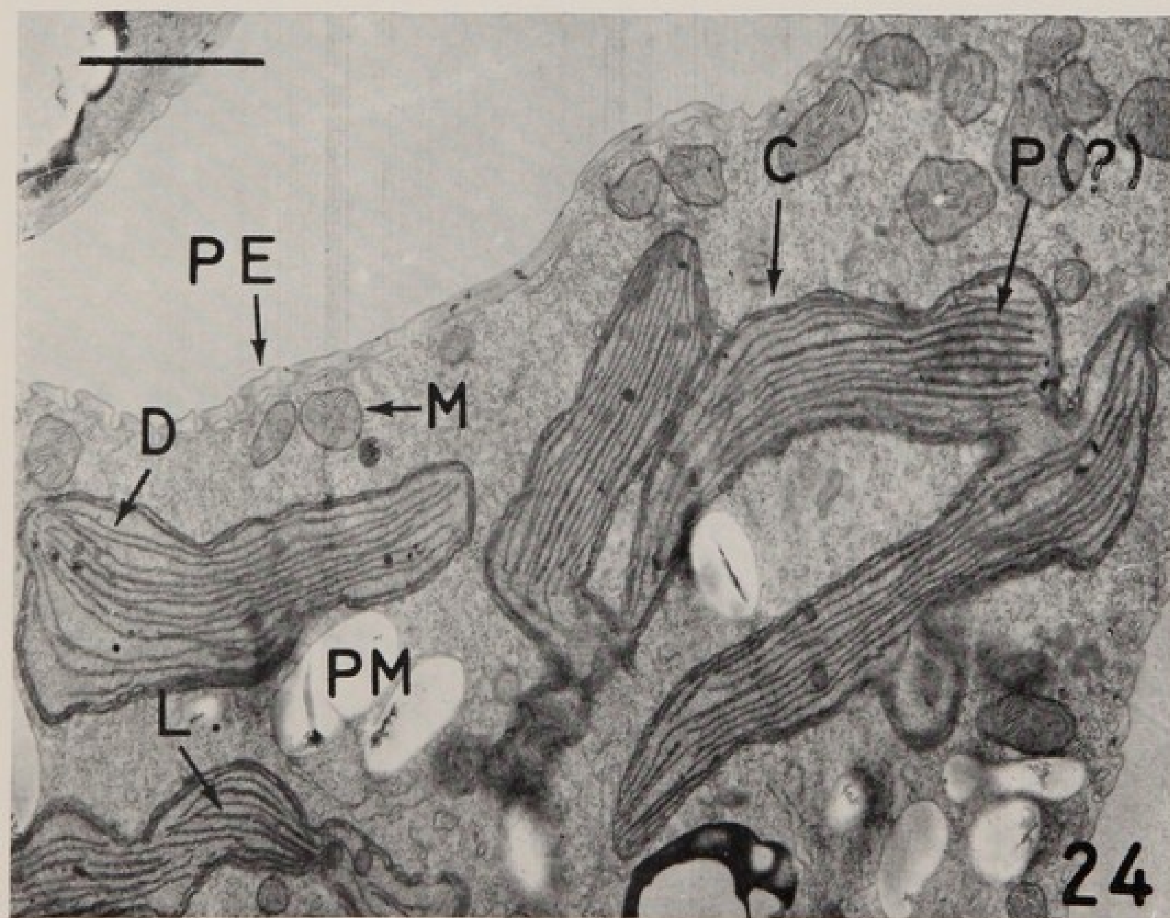

M
PM
C
L+D
ER
22

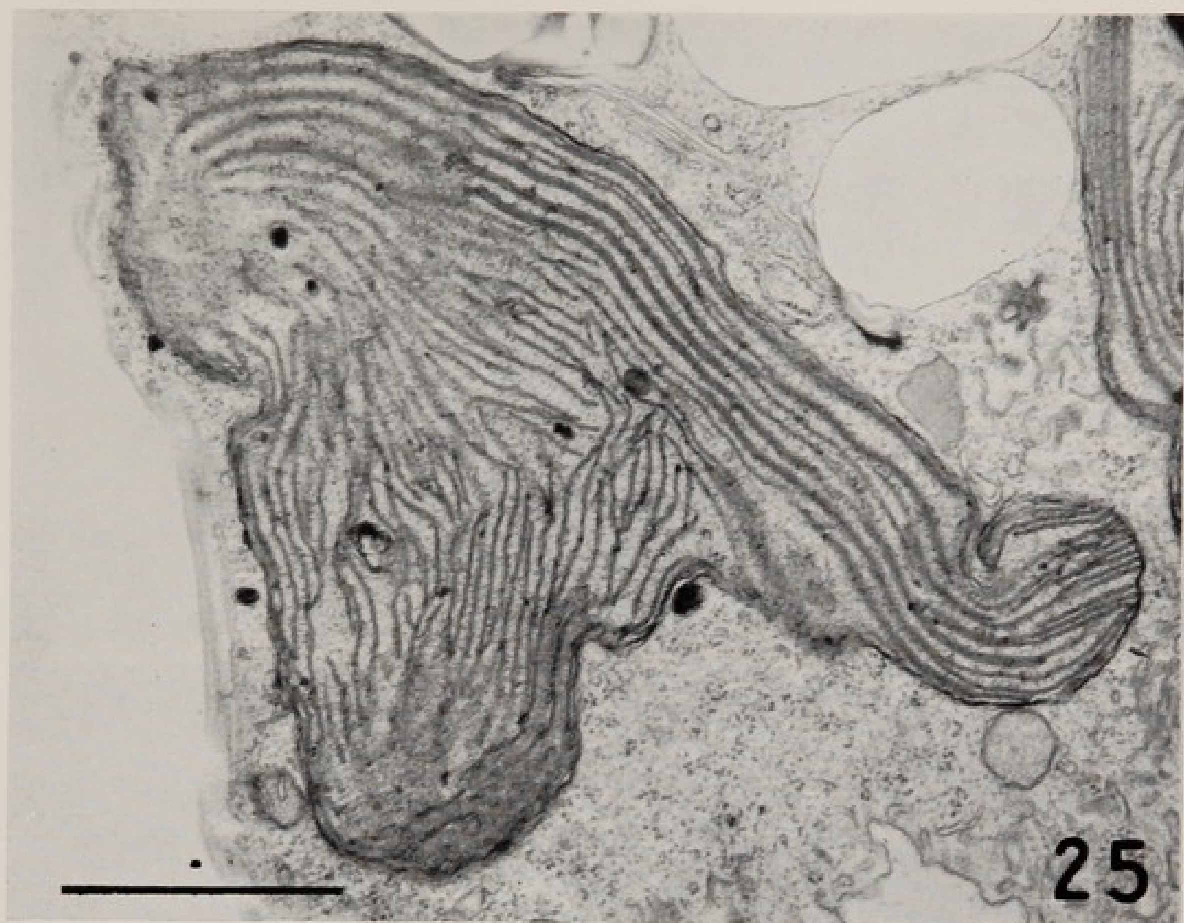
Fig. 23. Proplastids of O_1BS, an olive-green spontaneous mutant of *Euglena* grown in the dark. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

Fig. 24. Plastids of O_1BS, an olive-green spontaneous mutant of *Euglena* grown in the light. Note junctions between plastids on right. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)


M
D
N
PP
23


C
P(?)
PE
M
D
PM
L
24

FIGS. 25 and 26. Two representative examples of plastids in O_2BX, an olive-green X ray-induced mutant of *Euglena* grown in the light. (Liss *et al.*, 1965.) (Key to abbreviations in the legend of Fig. 1.)


25

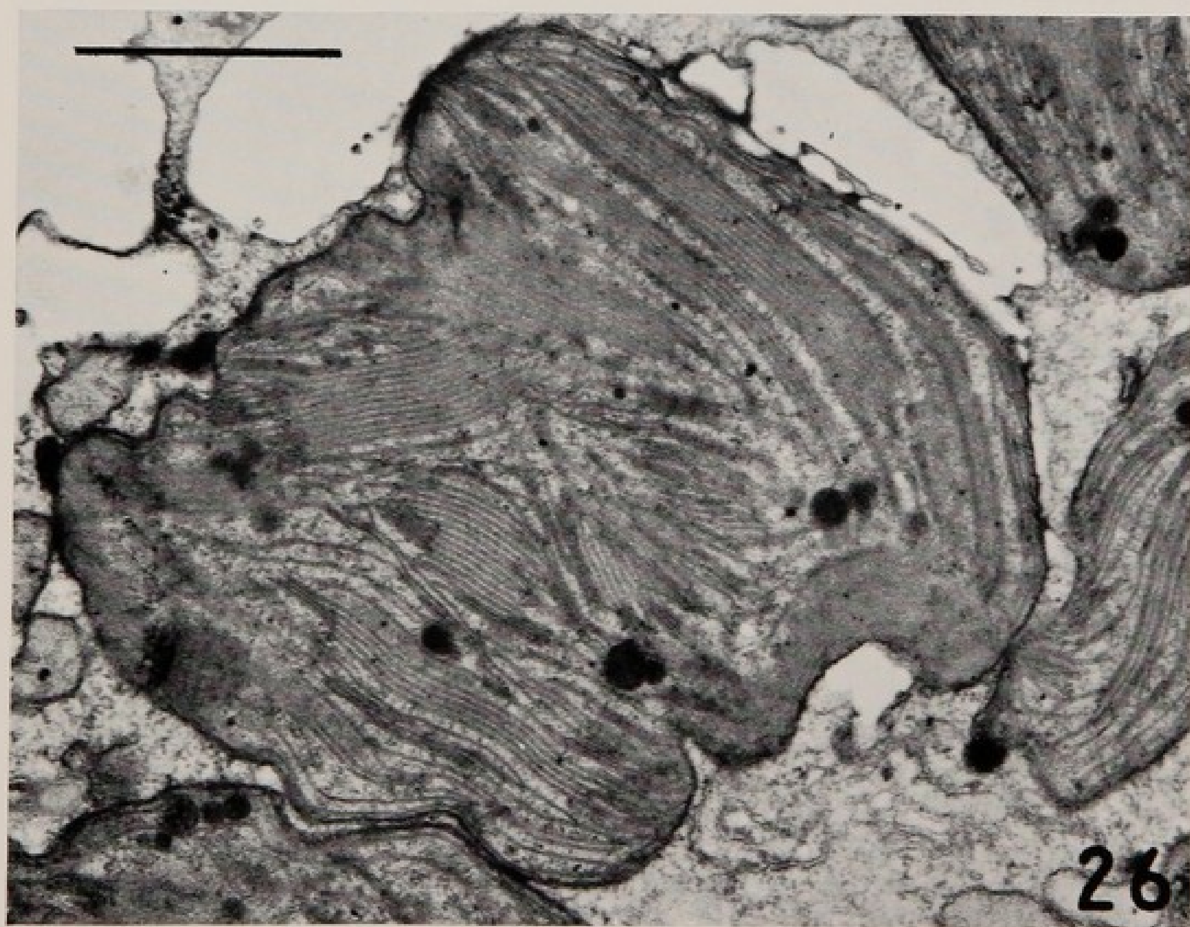
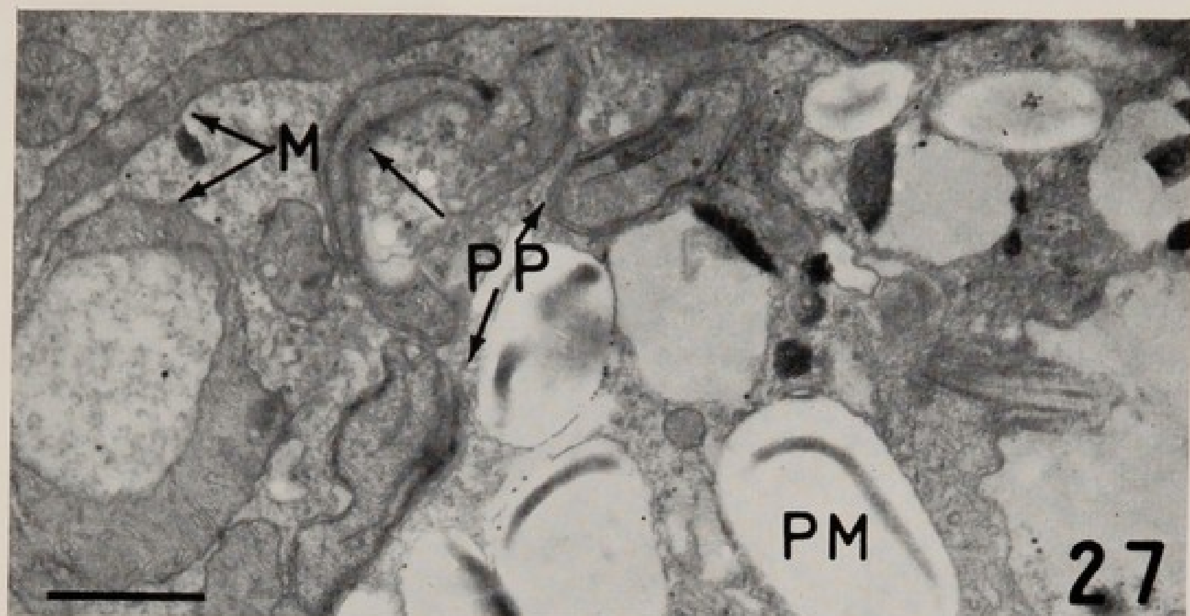
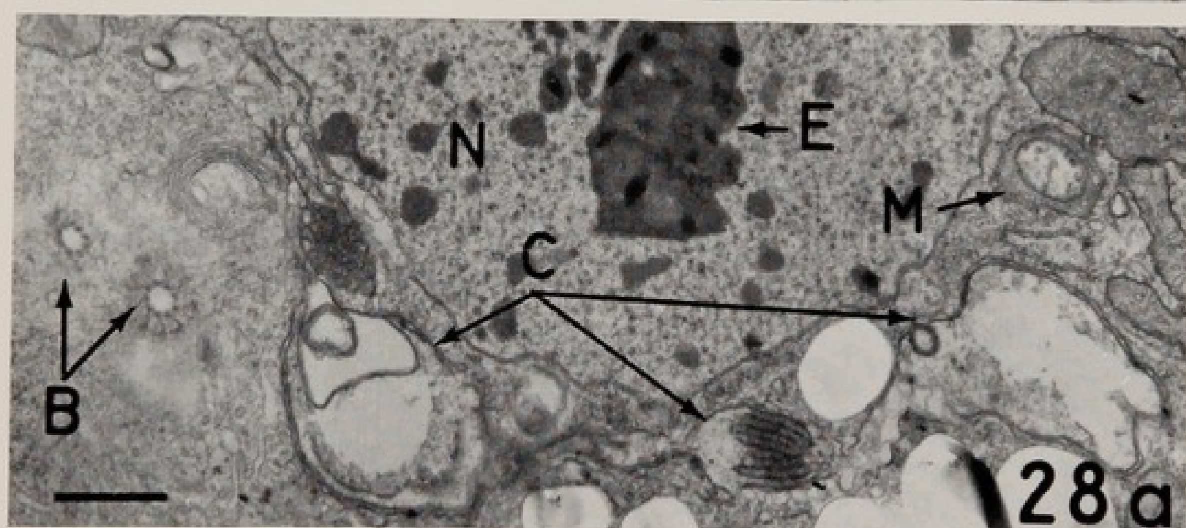

26

Fig. 27. Proplastids of Y_1BXD, a yellow X ray-induced mutant of *Euglena* grown in the dark. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

Figs. 28a and 28b. Representative plastids of Y_1BXD, a yellow X ray-induced mutant of *Euglena* grown in the light. Note that the limiting membrane of the three plastids in Fig. 28a is continuous. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)


M
PP
PM
27


N
E
M
C
B
28 a

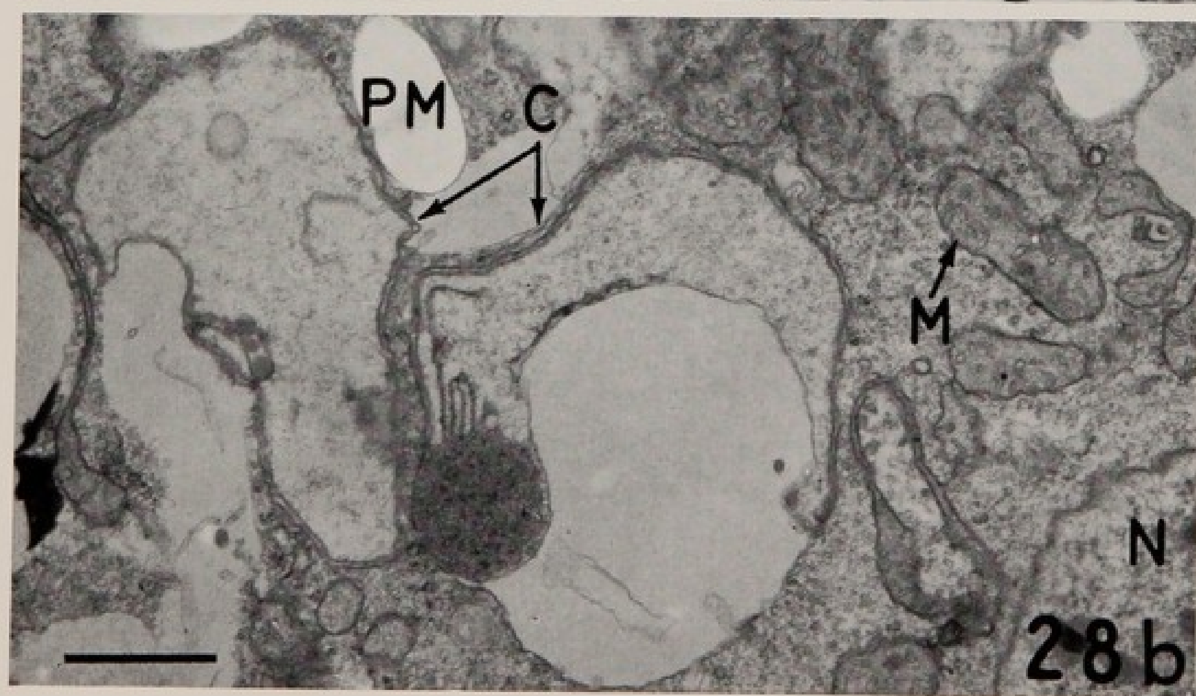
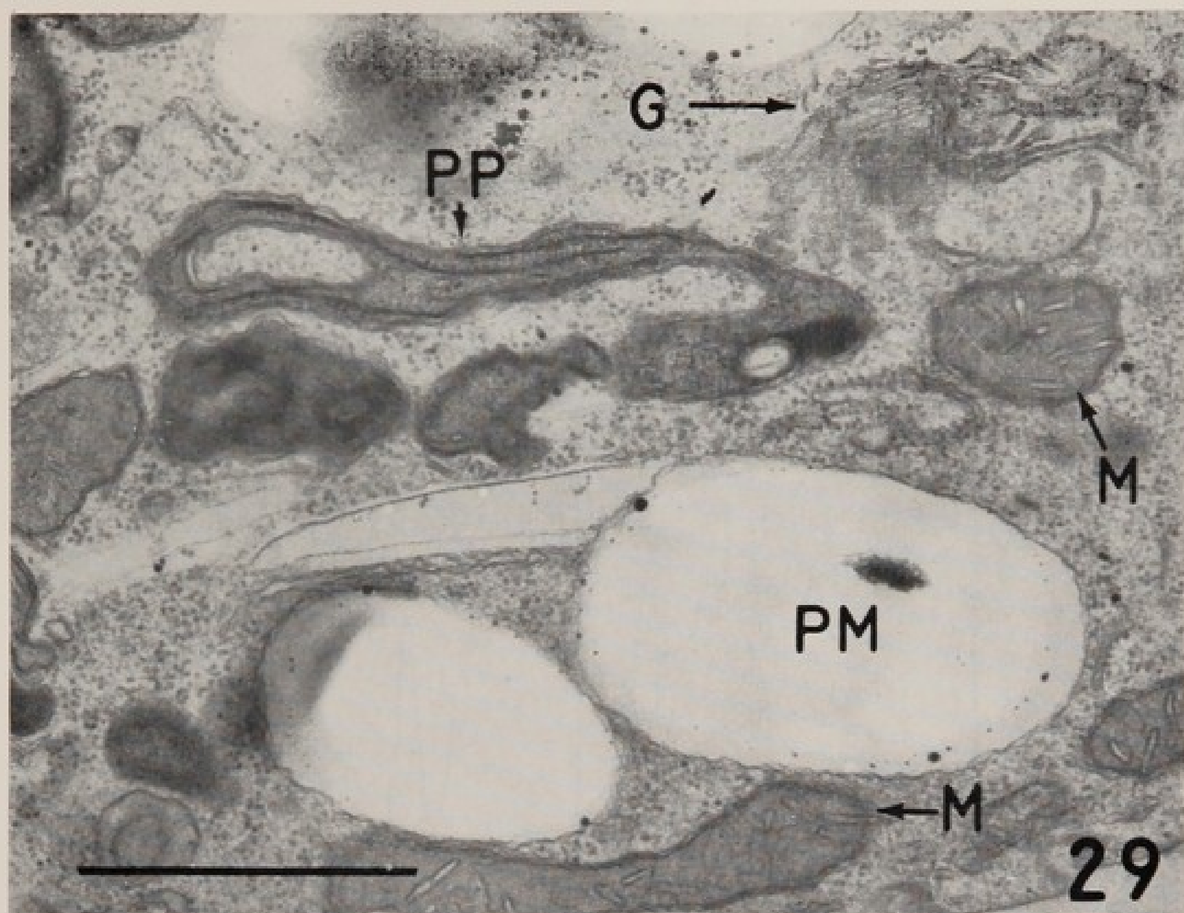
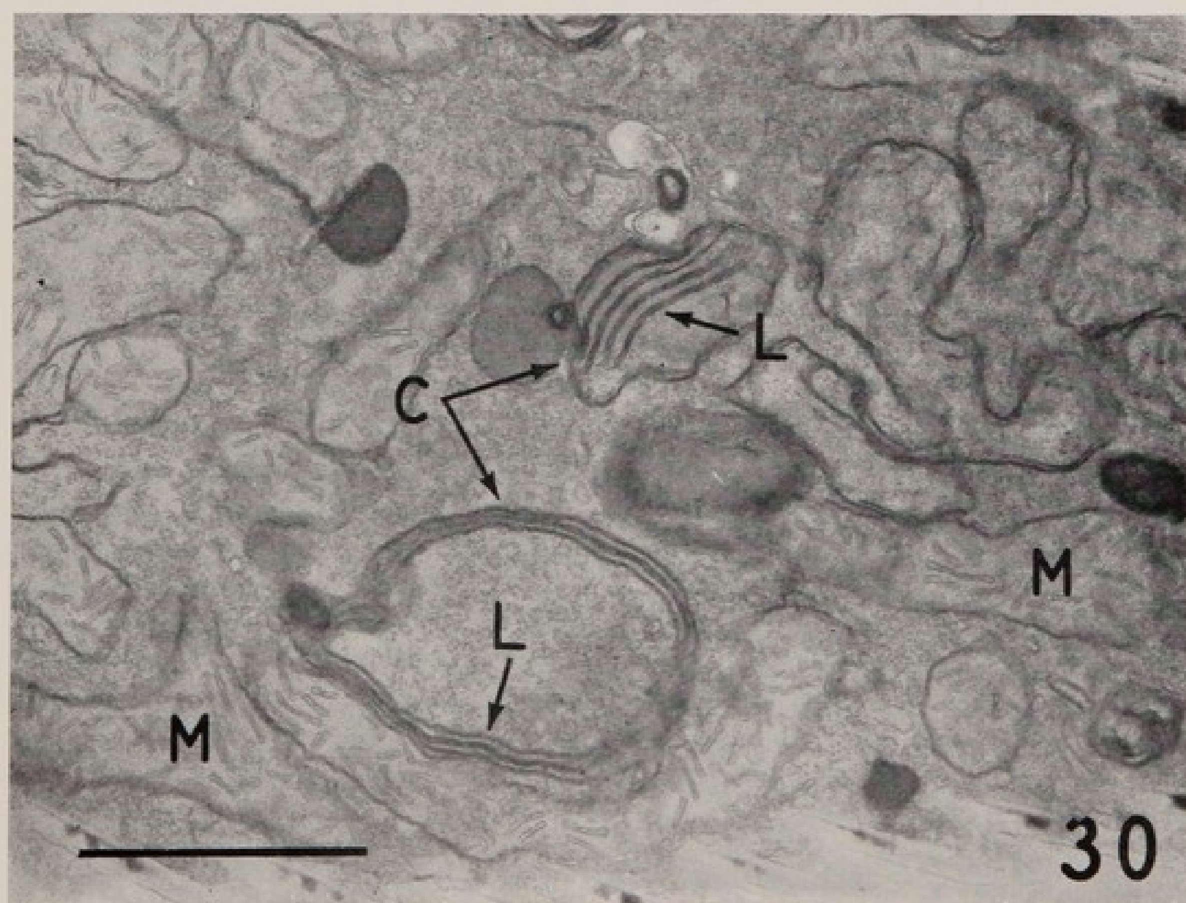

PM
C
M
N
28 b

FIG. 29. Proplastids of Y_3BUD, a yellow ultraviolet-induced mutant of *Euglena* grown in the dark. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)

FIG. 30. Plastids of Y_3BUD, a yellow ultraviolet-induced mutant of *Euglena* grown in the light. (Liss *et al.*, 1965.) (Key to abbreviations in legend of Fig. 1.)


G
PP
M
PM
M
29


L
C
M
L
M
30

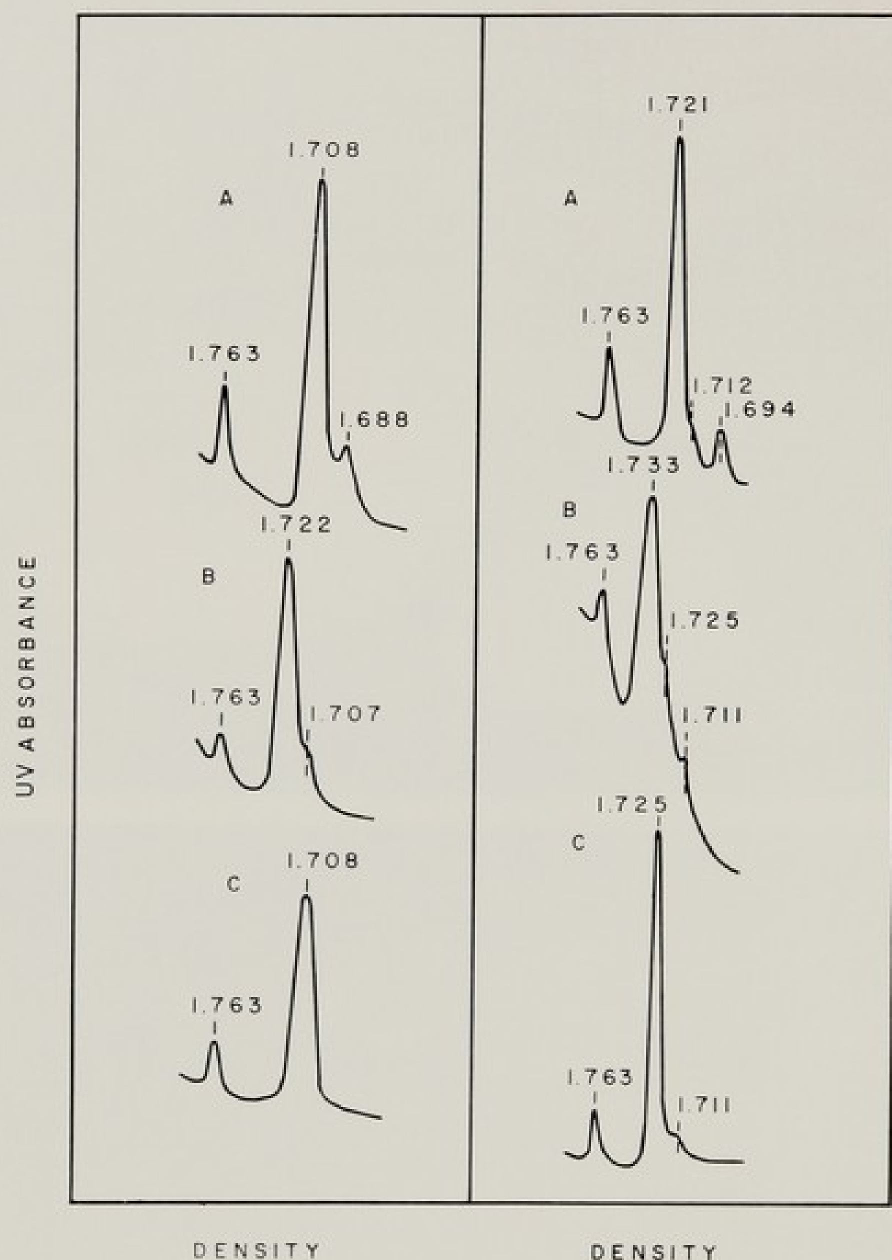


FIG. 31. Left: Microdensitometer tracings of ultraviolet photographs of DNA from *Euglena gracilis* var. *bacillaris* banded in cesium chloride density gradients. A, Light-grown wild-type cells, native DNA (13 μg); B, Light-grown wild-type cells, heat denatured DNA (13 μg); C, W_3BUL, native DNA (11 μg) (mutant lacking chloroplasts produced by treatment with ultraviolet). Right: Microdensitometer tracings of ultraviolet photographs of DNA banded in cesium chloride density gradients. A, *Chlamydomonas Reinhardi* Y_1, native DNA (6.4 μg); B, *Chlamydomonas Reinhardi* Y_1, heat-denatured DNA (6.4 μg); C, *Polytoma obtusum*, native DNA (7 μg).

In all cases, the band of density 1.763 is an added DNA of known density to calibrate the gradient. (Leff *et al.*, 1963.)

This interpretation was further strengthened by the finding that dark-grown cells in *Euglena* which contain proplastids also contain the 1.688 satellite band (Fig. 32) (Edelman *et al.,* 1964).

Soon after this initial demonstration of satellite DNA, it became possible to show that the 1.688 satellite was highly enriched in the chloroplast fraction of light-grown cells of both the *bacillaris* (Fig. 33) (Edelman *et al.,* 1964; Ray and Hanawalt, 1964) and Z strains (Brawerman and Eisenstadt, 1964b).

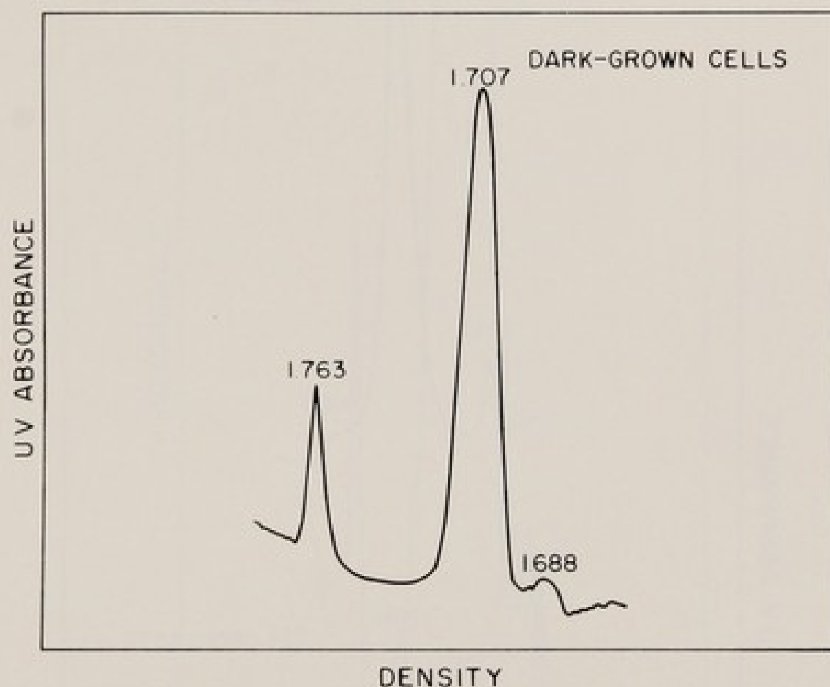


FIG. 32. Microdensitometer tracing of ultraviolet photographs of DNA from dark-grown wild-type cells of *Euglena gracilis* var. *bacillaris*. The band at 1.763 is added DNA of known density to calibrate the gradient. (Edelman *et al.,* 1964.)

It then became apparent that there were actually two DNA satellites in light-grown and dark-grown cells of wild-type *Euglena* (Fig. 34) (Edelman *et al.,* 1965; Ray and Hanawalt, 1965). The satellite at density 1.691 had been overlooked previously because the concentrations of DNA used in ultracentrifugation had been too low to permit its detection in the ultraviolet mutant cells. Wild-type *Euglena* cells contain, therefore, three types of DNA; main band DNA (1.707) which is associated with the nucleus, and two satellites: S_c (1.686) associated with the chloroplast fraction and with the ability of the cells to make chloroplasts, and another satellite S_x (1.691) which is associated with the small particle fraction of the cells containing the mitochondrial cytochromes (Edelman *et al.,* 1965). As may be seen from Fig. 35 (column A, unstarred), all strains of *Euglena* capable

of forming proplastids or a partial chloroplast, contain S_c in addition to S_x and main band. Treatment of any of these strains with ultraviolet and isolation of colorless clones results in a loss of S_c (Fig. 35, column A, starred). All strains incapable of forming plastid structures lack S_c but contain S_x and main band (Fig. 35, column B, unstarred). As expected,

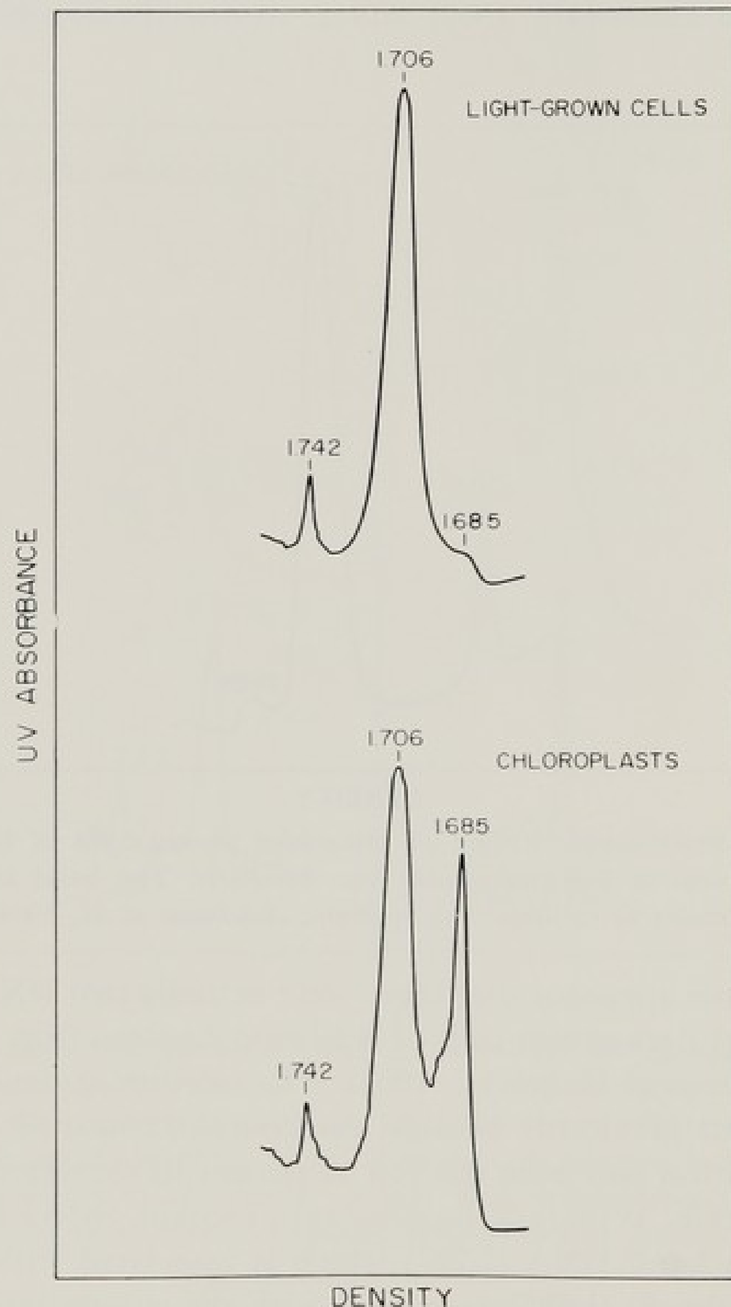


Fig. 33. Enrichment of satellite DNA of density 1.685 in the chloroplast fraction of light-grown cells of *Euglena gracilis* var. *bacillaris* compared with whole cell DNA. The curves show microdensitometer tracings of the ultraviolet absorption photographs of DNA separated in cesium chloride density gradients. In both cases, 1.742 represents added DNA of known density to calibrate the gradient. (Edelman *et al.*, 1964.)

treatment of these cells with ultraviolet produces no further alteration. This demonstrates that S_c is definitely correlated with the cells' ability to produce proplastids and chloroplasts and is consistent with the cytological evidence presented before for plastid loss in mutant cells produced by ultraviolet (Edelman *et al.*, 1965).

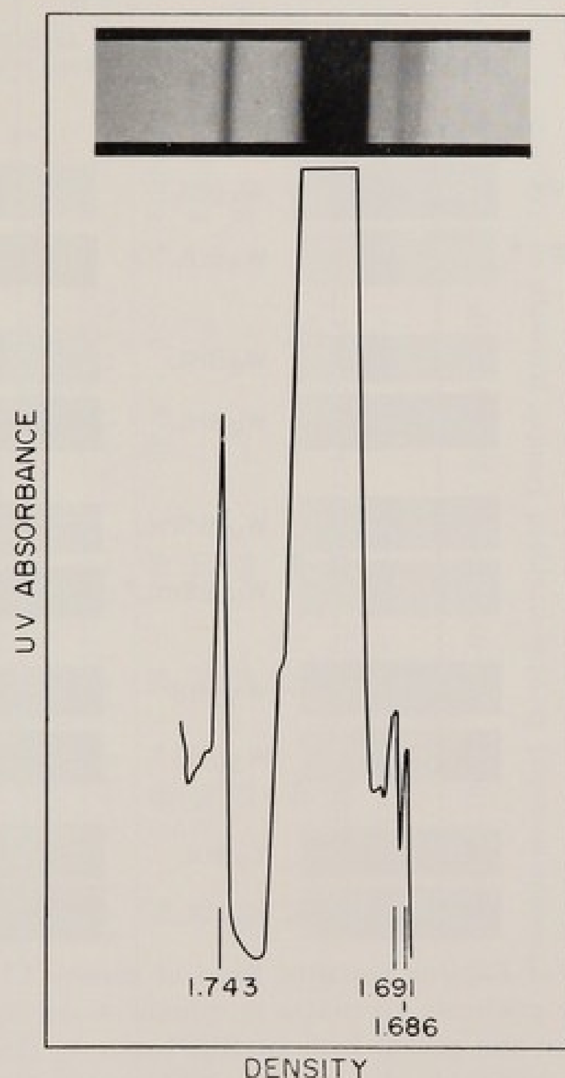


Fig. 34. Microdensitometer tracing of ultraviolet absorption photograph of DNA from light-grown cells of *Euglena gracilis* var. *bacillaris* separated on a cesium chloride density gradient. The moderate overloading of DNA in the gradient permits the resolution of two satellites. The band at 1.743 is added density standard DNA to calibrate the gradient. The overloaded band is main band DNA. (Edelman *et al.*, 1965.)

Table I summarizes the properties of the three types of DNA from *Euglena* cells. The *bacillaris* and Z strains are quite similar in densities to those found for main band and S_c. In *bacillaris*, the base compositions and molecular weights as isolated vary considerably among the three types of DNA.

Consistent with these findings, bromouracil incorporation by *Euglena* brings about an increase in plastid mutations (Scher and Collinge, 1965). Azathymine incorporation affords ultraviolet protection, presumably by reducing the probability of thymine dimer formation (Lyman and Smillie, 1963).

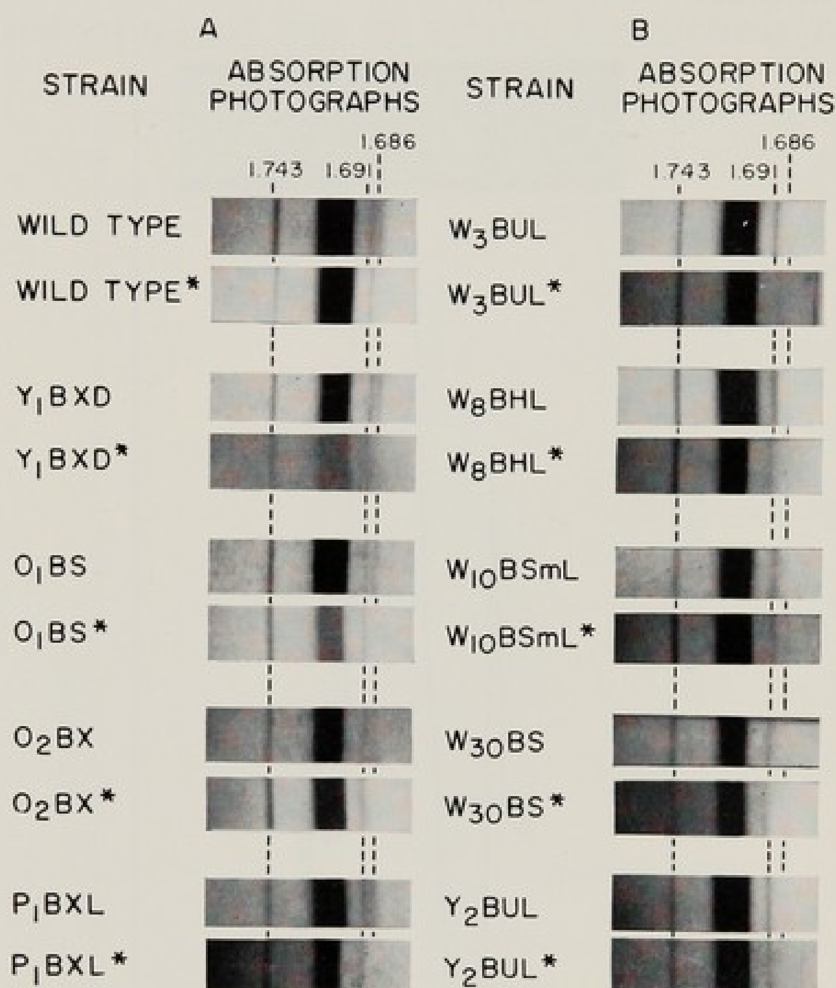


FIG. 35. The DNA's of *Euglena* mutants. Band of density 1.743 is density standard DNA added to calibrate gradient. All strains in column A are capable of developing at least a partial chloroplast and possess both satellite bands, S_x (1.691) and S_c (1.686). All strains in column B are incapable of even partial chloroplast development and contain only satellite band S_x (1.691). All strains in both columns A and B when subjected to treatment with ultraviolet light (indicated by asterisk) yield strains which lack S_c (1.686) but retain S_x (1.691). (Edelman *et al.*, 1965.)

F. *Organelle-Associated DNA in Other Species*

Since the original demonstration by Ris and Plaut (1962) of DNA fibrils in chloroplasts, satellite DNA's from many chloroplast-containing species have been described. These are summarized in Table II. As may be seen, the base compositions of satellite DNA's attributed to the chloroplast vary widely from species to species as do the main band

TABLE I
DNA OF *Euglena gracilis*

Source and method	Main band		S_c		S_x		Reference
	A+T	G+C[a]	A+T	G+C	A+T	G+C	
Bacillaris (density)	52	48	74	26	69	31	Edelman *et al.*, 1964, 1965
Bacillaris (thermal denaturation)	45	55	70	30	—	—	Edelman *et al.*, 1964
Bacillaris (analysis)	47	53	76	24	—	—	Ray and Hanawalt, 1964
Z strain (density)[b]	51	49	76	24	67	33	Brawerman and Eisenstadt, 1964b
Z strain (analysis)	49	51	75	25	—	—	Brawerman and Eisenstadt, 1964b
Z strain (thermal denaturation)[b]	47–52	48–53	74–79	21–26	—	—	Brawerman and Eisenstadt, 1964b
Molecular weight, as isolated (sedimentation)	$20–40 \times 10^6$		$20–40 \times 10^6$		$2.6–3.6 \times 10^6$		Ray and Hanawalt, 1964, 1965
Denaturation studies	Double-stranded		Double-stranded		Double-stranded		Edelman *et al.*, 1965
Density (gm/cm^3)	1.707		1.686		1.691		See above references

[a] Includes approximately 2.3% methyl cytosine (Ray and Hanawalt, 1964; Brawerman *et al.*, 1962b).
[b] Calculated from data given in reference.

TABLE II

DNA of Chloroplast-Containing Species and Related Organisms

Group	Species	Main band[a]			Satellites						References
		Density	A+T	G+C	Density	A+T	G+C	Density	A+T	G+C	
Euglenophyta	*Euglena gracilis* var. *bacillaris*	1.707	52	48	1.686[b]	74	26	1.691[c]	69	31	Table I
	Euglena gracilis var. Z	1.708	51	49	1.684[b]	75	25	1.692	67	33	Table I
Chlorophyta	*Chlamydomonas Reinhardi*	1.726(?)	38	62	1.702[b,d]	61	39	—	—	—	Sager and Ishida, 1963
	Chlamydomonas Reinhardi	1.723	36	64	1.695[e]	64	36	—	—	—	Chun *et al.*, 1963
	Chlamydomonas Reinhardi	1.721	38	62	1.694[e]	65	35	1.712	47	53	Leff *et al.*, 1963
	Polytoma obtusum	1.725	35	65	—	—	—	1.711	48	52	Leff *et al.*, 1963
	Chlorella ellipsoidea	1.716	43	57	1.695[b]	64	36	—	—	—	Chun *et al.*, 1963; Iwamura and Kuwashima, 1964; Iwamura, 1960

TABLE II (*Continued*)

Group	Species	Main band[a]			Satellites						References
		Density	A+T	G+C	Density	A+T	G+C	Density	A+T	G+C	
Chrysophyta	*Ochromonas danica*	1.708	52	48	1.691	69	31	—	—	—	Edelman *et al.*, 1963
Tracheophyta	*Beta vulgaris*	1.695	64	36	1.705[c]	54	46	1.719[e]	40	60	Chun *et al.*, 1963
	Spinacia oleracea	1.695	64	36	1.705[e]	54	46	1.719[e]	40	60	Chun *et al.*, 1963
	Vicia faba	A:G = 1.54			A:G = 1.67[b]			—	—	—	Kirk, 1963

[a] In all cases, main band DNA is attributed to the nucleus.
[b] Shown to be associated with chloroplasts.
[c] Attributed to mitochondria.
[d] Possible error in calculation of density in original paper.
[e] Attributed to chloroplasts.

TABLE III

Species Lacking Chloroplasts but Containing DNA Satellites

Group	Species	Main band			Satellites			Reference
		Density	A + T	G + C	Density	A + T	G + C	
Vertebrates	Mouse (fibroblast)	1.702	57	43	1.691	68	32	Kit, 1961; Chun and Littlefield, 1963
	Calf (thymus)	1.699	60	40	1.713	46	54	Schildkraut *et al.*, 1962
	Guinea pig	1.697	62	38	1.703	56	44	Kit, 1961
	Chick (heart, liver)	1.698	61	39	1.707[a]	52	48	Rabinowitz *et al.*, 1965
	Salmon (sperm)	1.703	56	44	1.688	71	29	Schildkraut *et al.*, 1962
Arthropods	*Cancer* (7 species)	1.699- 1.701	41- 43	57- 59	1.677- 1.683	1- 3	99- 97	Sueoka, 1961 M. Smith, 1964
	Balanus nubilis	1.706	53	47	1.714	45	55	M. Smith, 1964
Protozoa	*Paramecium aurelia*	1.689	70	30	1.696[b]	63	37	Smith-Sonneborn *et al.*, 1963
	Blastocrithidia culicis	1.715	44	56	1.696	63	37	Mandel, 1965
	Crithidia fasciculata (Anoph.)	1.713	46	54	1.698	61	39	Mandel, 1965
	Crithidia fasciculata (Culex)	1.717	42	58	1.693	66	34	Schildkraut *et al.*, 1962
	Crithidia oncopelti	1.709	50	50	1.699 1.691[c]	60 68	40 32	Marmur *et al.*, 1963
	Naegleria gruberi S	1.693	66	34	1.683 1.702	76 57	24 43	Mandel, 1965
	Leishmania enrietti	1.721	43	57	1.699[e]	64	36	DuBuy *et al.*, 1965

TABLE III *(Continued)*

Group	Species	Main band			Satellites			Reference
		Density	A + T	G + C	Density	A + T	G + C	
Fungi	*Physarum polycephalum*	1.703	56	44	1.714	45	55	Braun *et al.*, 1965
	Blastocladiella emersonii	1.725	34	66	1.710	49	51	Comb *et al.*, 1964
	Neurospora crassa	1.712	47	53	1.701[a]	58	42	Luck and Reich, 1964
Bacteria	*Halobacterium* (2 species)	1.727	32	68	1.718	41	59	Joshi *et al.*, 1963
	Serratia marcescens	1.718	42	58	1.709[d]	50	50	Marmur *et al.*, 1961
					1.703[d]	56	44	
	Proteus mirabilis	1.698	61	39	1.710[d]	49	51	Wohlhieter *et al.*, 1964

[a] Attributed to mitochondria.
[b] Attributed to ϰ particles.
[c] Attributed to an endosymbiont.
[d] Attributed to an episome.
[e] Attributed to the kinetoplast.

DNA's. Nass and Nass (1963) demonstrated DNA fibrils in mitochondria of chick embryo tissue, and the DNA was subsequently isolated by Rabinowitz *et al.* (1965). Luck and Reich (1964) have provided convincing evidence that this organelle is self-replicating in *Neurospora* and contains its own type of DNA. Table III summarizes the base compositions of satellite DNA's from species lacking chloroplasts.

G. *Localization of Information for Chloroplast Constituents*

The experiments reviewed above for *Euglena* and other species seem to leave no doubt that a DNA exists in the chloroplast and in other organelles such as mitochondria. The evidence from studies with ultraviolet on chloroplast continuity in *Euglena* are consistent with the interpretation that the information for the construction of the *Euglena* chloroplast or proplastid resides in the organelle itself. The existing data do not indicate, however, how much of the information is contained within the organelle. Since sexual fusion has not been observed in *Euglena* and attempts to find an infecting virus or transformation have been uniformly unsuccessful, it has been impossible to study the various modes of inheritance in *Euglena* in the usual way. In those species where this type of genetic analysis has been possible (e.g., maize and *Oenothera*) interrelationships between the nucleus and the chloroplast are strongly suggested by the data. All we can say at present is that at least part (a crucial part) of the information required to construct a chloroplast or proplastid resides in the organelle itself. It is possible that varying degrees of organelle autonomy exist in various species. If appropriate genetic and biochemical techniques become available it may be possible to assess whether the complete informational autonomy of the plastids (which is consistent with the present data) is indeed true in organisms such as *Euglena* or whether some genetic interaction with the nucleus is present as in higher plants. At the very least, the replication of organelles such as the chloroplast is responsive to overall signals for cell division which coordinate the division of the cytoplasmic organelles, nucleus, and of the cell itself.

IV. Possible Control Mechanisms for Chloroplast Development and Replication

Figure 36 shows a model which we have constructed to relate the various facts known about chloroplast development and replication in *Euglena*. At worst it is a useful mnemonic device; at best it may serve as a working hypothesis. At the left are the dark-grown cells which have about 30

proplastids containing protochlorophyll(ide) and DNA. Maintained in darkness, these cells divide and the proplastids replicate to keep the numbers constant from one generation to the next. This implies that the DNA contained in each proplastid replicates itself in each cell generation, presumably via a DNA polymerase enzyme contained in the plastid itself. We assume that small amounts of this enzyme are present during growth in the dark to allow the continuity of the proplastid in dark-grown cultures.

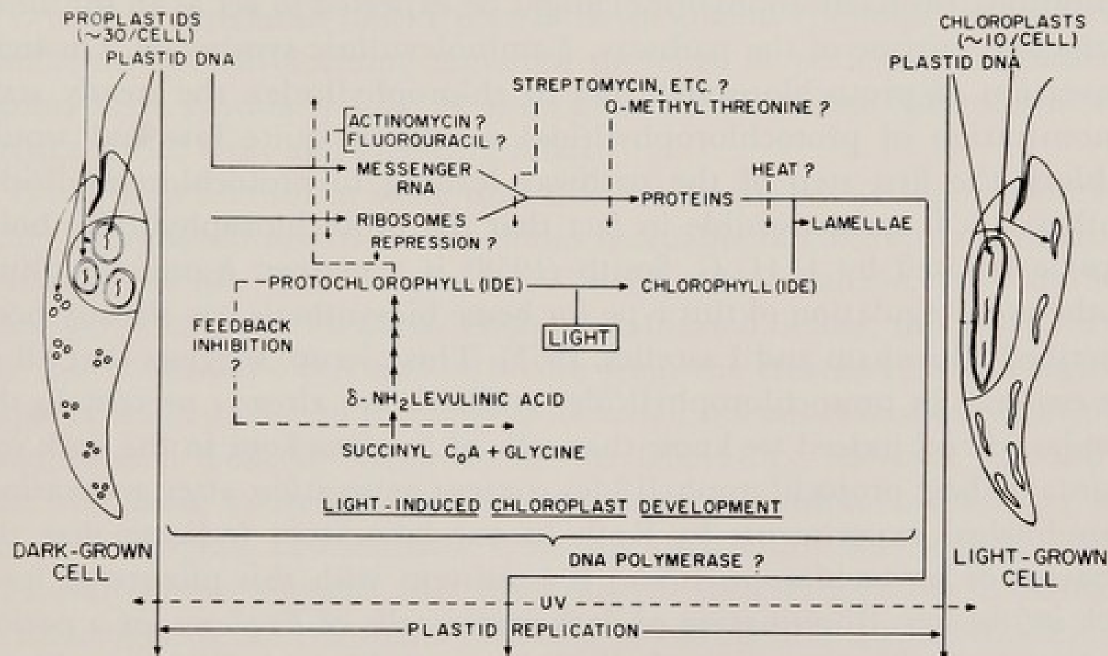


FIG. 36. Hypothetical model for control of chloroplast development and replication in *Euglena*. The details are explained in the text.

Similarly, at the right of the diagram, is the light-grown cell which contains about 10 chloroplasts. Since both dark-grown and light-grown cells have multiplicities of about 30 for ultraviolet inactivation of replication, we assume that 3 proplastids have fused during the transition from dark to light with conservation of the DNA. Each chloroplast then would contain three of the DNA units formerly present in each proplastid. When the chloroplast divides during light growth of the cells, these DNA's are replicated as in the dark-grown cells. Since our experiments clearly showed that ultraviolet specifically blocks the transmission of plastid-forming ability to daughter cells at the time of cell division we have indicated the site of action of ultraviolet as being on DNA replication.

Now let us consider the developmental process from proplastid to chloroplast during light induction. The proplastids contain small amounts of protochlorophyll(ide) (about 1/5,000 the amount of pigment compared with the chlorophyll which will eventually be formed). It is

well known that the action spectrum for light induction of chloroplast formation from proplastids in *Euglena* and higher plants is the absorption spectrum of protochlorophyll(ide) and that the light-induced conversion of protochlorophyll(ide) to chlorophyll(ide) is a necessary step in chlorophyll biosynthesis. The small amounts of protochlorophyll(ide) present (much less than would be expected from an intermediate accumulating behind a dark block) suggest that the formation of protochlorophyll in dark-grown cells is under feedback control. By analogy to other feedback inhibitions, protochlorophyll(ide) might be expected to act as an inhibitor of the first enzyme of the pathway, δ-aminolevulinic synthetase. On light conversion of protochlorophyll(ide) to chlorophyll(ide), the steady state concentration of protochlorophyll(ide) would be quite low and would unblock the first step of the pathway leading to protochlorophyll(ide) synthesis. It is quite possible in fact that the protochlorophyll(ide) holochrome isolated by J. H. C. Smith (1958) is a masked δ-aminolevulinic synthetase. Regulation of this type for heme biosynthesis has already been described (Burnham and Lascelles, 1963). This picture suggests that all of the enzymes in protochlorophyll(ide) synthesis are already present in the proplastid and indeed we know that cells of *Euglena* kept in the dark can maintain their protochlorophyll(ide) content generation after generation. Unpublished experiments by Roth in our laboratory indicate that the behavior of protochlorophyll(ide) is consistent with this picture of feedback inhibition. Illumination of dark-grown cells of *Euglena* for a period of time results in conversion of all of the protochlorophyll(ide) to chlorophyll(ide). If left in the dark, these cells will resynthesize protochlorophyll(ide) to approximately the same level as before and then cease. After conversion with light again, the same thing occurs. This can be done many times in succession suggesting that light unblocks protochlorophyll(ide) synthesis by converting the inhibitory protochlorophyll(ide) to chlorophyll. Resynthesis of protochlorophyll(ide) then occurs until the original level is reached and the biosynthesis is turned off once more. Carell and Kahn (1964) have described some enzymes of porphyrin synthesis in *Euglena*.

The illumination of protochlorophyll(ide) does far more than mediate chlorophyll production. As described earlier, light induction results in the formation of many chloroplast-specific proteins. This suggests that the conversion of protochlorophyll(ide) to chlorophyll(ide) results in the derepression of plastid DNA permitting the synthesis of RNA messengers. Evidence for template RNA in chloroplasts has appeared (Brawerman and Eisenstadt, 1964a). Consistent with this is the finding that fluorouracil (Smillie, 1963) inhibits chloroplast development if given sufficiently soon

after light induction. The system becomes insensitive to fluorouracil after about 24 hours of development indicating that all of the necessary RNA has been synthesized by this time. Actinomycin is also partially inhibitory (B. G. T. Pogo and Pogo, 1964; McCalla and Allan, 1964). As noted before, a unique species of ribosome exists in *Euglena* plastids (Brawerman, 1963). Thus, if messenger is produced from plastid DNA it could associate with plastid ribosomes to form a protein synthesizing system. Hadacidin, an inhibitor of adenine biosynthesis, blocks chloroplast development if given within the first 24 hours of development (Mego, 1964).

O-methyl threonine blocks plastid development (perhaps by replacing isoleucine, thereby forming nonsense proteins, or perhaps by inhibiting amino acid synthesis) and is reversible by isoleucine (Aaronson and Bensky, 1962; Gray and Hendlin, 1962).

It has been known for some time that treatment with streptomycin and other compounds results in cell clones incapable of chloroplast formation (Provasoli *et al.*, 1948; Rosen and Gawlik, 1961; Zahalsky *et al.*, 1962; Ebringer, 1962a,b,c; McCalla, 1962). As indicated before, this results from the loss of plastid DNA and structure from the treated cells during subsequent cell division. In this respect its action resembles that of ultraviolet. Unpublished work by Wilensky in our laboratory, however, has shown that the same concentrations of streptomycin, unlike ultraviolet, also block chloroplast development from the proplastid. The evidence from bacterial systems suggests that streptomycin interferes at the attachment of messenger to the ribosomes resulting in coding ambiguities (Spotts and Stanier, 1961; Cox *et al.*, 1964; Davies *et al.*, 1964). Applied to the *Euglena* chloroplast system we might suppose that streptomycin's primary action is to block chloroplast-specific protein synthesis in a similar manner. If one of the proteins produced by this system were the DNA polymerase for the plastid DNA, then interference with development by streptomycin might result in eventual loss of plastid DNA through lack of enough enzyme for its replication. Streptomycin would therefore eventually lead to complete elimination of the plastid structures and DNA from the cells as observed.

Temperatures above 34°C also result in plastid and plastid DNA elimination from *Euglena* cells (Pringsheim and Pringsheim, 1952; Brawerman and Chargaff, 1960). It is possible that some enzymes involved in DNA replication in the plastid are sensitive to denaturation at these relatively low temperatures.

In short, there seems to be no reason not to consider chloroplast induction by light as a complex coordinated type of enzyme induction employing the usual translation and transcription mechanisms described for

other systems. As with the most favorable systems for study (bacteria, particularly, *E. coli*) our greatest ignorance is of the mechanisms of repression, derepression, and control of protein synthesis.

V. Speculations Concerning the Evolution of the Chloroplast

Having provided a positive answer to the question of chloroplast autonomy through replication we confront the question of the origin of the organelle being replicated. Basically, we have the two alternatives often voiced for the origin of viruses. The first alternative visualizes that a portion of the nuclear DNA became detached as a separate entity sometime in the distant past. This episome then proceeded to replicate in the cytoplasm independently of the nucleus and eventually evolved into the structure we call a chloroplast.

The second alternative supposes that at some point in evolution cells resembling primitive animal cells and primitive photosynthetic bacteria or blue-green algae existed contemporaneously. Primitive photosynthetic cells of this type invaded the envelopes of the primitive animal-like cells and established themselves as endosymbionts. Eventually these photosynthetic invaders lost their cell walls, and perhaps to some extent became nutritionally dependent for growth factors on the host cell. Replicating along with the host they gradually became subject to precise control of division by the host cell, and became habituated as chloroplasts.

There is very little evidence for choosing among these alternatives (and the many others which could be proposed). The fact that chloroplast DNA's of many species are so different in base composition from their cells' nuclear DNA would speak against the first alternative, but arguments could be advanced that the nuclear DNA of average composition could be quite heterogeneous along its length so that a detached piece might have a different base composition from the average for the nuclear DNA as a whole. Also, we could argue that the episomal DNA, having become detached, could continue to be changed in composition through evolution resulting in a different base composition from the nucleus.

In regard to the second alternative, there would be no problem of DNA composition since the invader would not have to have the same DNA composition as the host. Also, it is possible to discern possible steps in the habituation of endosymbionts occurring today. Many cases of endosymbiotic algae inhabiting animal cells are known. In these cases the endosymbiont retains its wall and can often be grown independently of the host. In the case of *Cyanophora paradoxa* (Hall and Claus, 1963) a blue-green alga has become habituated in an animal cell, has lost its wall, and divides along with the host. It is a small step from here to becoming an organelle.

The heterogeneity of base composition of chloroplast DNA's (see Table II) among several species, however, would indicate that if their origin were through invasion and habituation, there must have been several different cases of invasion rendering the origins of chloroplasts in different species polyphyletic. It is possible, of course, that there was only one invasion and that subsequent evolution has changed the base composition of the invader in different host cells.

Although one cannot choose among these and other alternatives, any comprehensive theory of the origin of life and its connection with cellular evolution must eventually account for the origin and evolution of autonomously replicating organelles.

VI. Conclusion

Much has been learned about chloroplast development and inheritance in many systems. The *Euglena* system has been particularly rewarding because chloroplast and proplastid replication are more sensitive to experimental interference than is cell viability. With the findings that chloroplast replication and development involve the replication and reading of an autonomous code, this area of development has entered the mainstream of molecular biology only to find itself confronted by this area's major problem, the mechanism of control. Perplexity likes company, however, and the interesting work is just beginning.

Note Added In Proof: The possibility exists that proplastids are already linked in the dark-grown cells, in groups of three, by fine membrane connections, but in the fluorescence microscope appear singular because the protochlorophyll(ide) is concentrated in the larger parts of the structure. If this were true, it would offer an alternative explanation to the fusion of proplastids postulated in this paper and could still be consistent with all the relevant data.

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Extrachromosomal Heredity in Fungi

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Introduction

Chromosomal genetic systems are highly unified in that they are definable by principles and common features that hold everywhere in the biological world, at least among eukaryotes. Even among prokaryotes, wherever much is known about them, as in certain bacteria, there is an obvious fundamental unity and a high degree of similarity to chromosomal genetic systems in general. In contrast, those seemingly rarer hereditary phenomena which have been called *extrachromosomal* or, more restrictedly, *cytoplasmic* heredity appear as a sprawling and now fairly large number of loosely connected observations. If we wish to preserve rigorous scientific caution, the only certain connection among these observations is the general conclusion that they do not seem to fit the pattern of chromosomal inheritance we have learned to expect. As will be seen, this common factor is the inevitable consequence of the techniques that have been used to detect extrachromosomal heredity. Under these circumstances, certain biologists have retained reservations about the reality of extrachromosomal heredity; others have questioned its significance; and many hold that the study of extrachromosomal heredity is still in a primitive and highly unsatisfactory state. One basis for the last mentioned view is that extrachromosomal hereditary systems as yet have no point of physical reference, or have at best a dubious one, of the kind provided by chromosomes for Mendelian heredity. Moreover, we can contend that extrachromosomal heredity, as we are able to view it today, lacks any clear set of underlying principles that allow valid inference regarding mechanism—principles of the kind provided by Mendel and his followers before proof of the chromosomal basis for Mendelian heredity was established.

As a matter of fact, we still cannot be certain that some single mecha-

nism, some single structure, or some single molecular species can be found —or even should be sought—to be the basis for extrachromosomal heredity. Indeed it seems unlikely, although still possible, that the basis for non-Mendelian plastid inheritance in higher plants will ultimately be found to be the same as that underlying the extrachromosomal hereditary phenomena in fungi around which the following discussion will center. When we turn to such apparently diverse instances as the killer phenomenon (Sonneborn, 1961) and cortical inheritance (Sonneborn, 1963), both of which occur in *Paramecium* and both of which have been called extrachromosomal heredity, the search for a single mechanism underlying extrachromosomal heredity seems likely to be chimerical. The first instance, the killer phenomenon, clearly depends upon the presence of a particulate, DNA-containing parasite. In the second instance, cortical configuration is unaffected by substitution either of the nucleus or of the cytoplasm from an animal characterized by an alternative configuration. It is as though the cortex itself carries hereditary information, perhaps of a structural kind, that serves as an indispensable configurational model for orientation of the products of nuclear gene action. It is scarcely justifiable, then, to minimize either the reality or the significance of cytoplasmic heredity because no set of unifying hypotheses has been developed that accounts for all instances, or because no single cellular structure seems likely to be a common vehicle of extrachromosomal heredity. The phenomena requiring explanation are most likely a conglomerate.

In spite of this, a unifying basis for the consideration and interpretation of extrachromosomal heredity may be found, and this basis may turn out to be the chromosomal genetic system. First, we can justifiably expect that all participants in the activities of a cell interact, and indeed interact in a coordinated way. Perhaps the various manifestations of extrachromosomal heredity will come to be best and most coherently understood in terms of their orderly interactions with the central genetic apparatus in the nucleus when these interactions are finally discovered and correctly interpreted. Second, the possibility remains that the primary genetic information accounting for most if not all instances of extrachromosomal heredity resides in the chromosomes. Nanney (1958) has pointed out most persuasively that most hereditary phenomena designated extrachromosomal may in fact be *epigenetic* rather than *genetic*. We now know enough about the maintenance of cellular properties to be aware that the expression and the persistence of certain properties are not directly attributable to the genes that provide the potential for their expression, but depend rather upon regulating mechanisms at the transcriptional or at the metabolic

level. In other words, phenotype as well as genotype may be inherited, and genetic analysis does not always serve to distinguish between the two.

The foregoing remarks might lead to the belief that extrachromosomal heredity has produced more semantic analysis than experimental analysis and that it cannot be adequately defined for purposes of serious consideration at this time. The author intends to convey no such impression. Under the "umbrella" of extrachromosomal heredity are a large number of facts derived from different studies of various organisms; these facts confront biologists and can scarcely be ignored. Furthermore, they pose intriguing problems that impinge upon fundamental biological phenomena. Among the unresolved problems recognizable by the geneticist today, those relating to extrachromosomal heredity appear highly significant. Their resolution is likely either to extend our concepts of genetic systems, or to enlarge our understanding of regulation and development, or both.

The purpose of this paper is to examine extrachromosomal heredity in ways that will permit some evaluation of the significance of its attributes and convey some impression of what we know and of what we do not know and must determine. For the most part, the discussion will be restricted to appropriate phenomena in fungi in order to keep it within reasonable bounds; and even for the fungi, no effort will be made to catalogue all of the instances of extrachromosomal heredity that have been reported.

Evidence for Extrachromosomal Heredity

Evaluation of the present status of extrachromosomal heredity requires an understanding of the evidence from which basic inferences have been made. In general, the existence of instances of extrachromosomal heredity has been established through contrast with chromosomal heredity, and the arguments are primarily genetic arguments. As an example for purposes of discussion, we may consider the SG system in *Neurospora* which has been investigated in our laboratory for several years. The phenotype of SG strains of *N. crassa* differs from that of normal (N) strains in that the spores, both conidia and ascospores, germinate appreciably more slowly. After an early period of slow growth, SG cultures become indistinguishable from normal cultures by any means that we have been able to devise.

The details of the facts that have led to the conclusion that SG represents an extrachromosomal property have been published elsewhere (Srb, 1958, 1963), but an outline review of the appropriate observations is relevant here. The first clue that SG may not be a chromosomally determined

attribute is its failure to show segregation at meiosis after crosses with normal *Neurospora* have been made. In addition, reciprocal crosses with N strains give different results. As shown in Fig. 1, sexual reproduction in *Neurospora crassa* is such that reciprocal crosses are possible. On appropriate media most strains develop both conidia (asexual spores) and protoperithecia (incipient fruiting bodies). Fertilization is carried out by placing conidia from a culture of one mating type, either *A* or *a*, on protoperithecia from a culture of the alternative mating type. Inside a protoperithecium nuclear fusions occur followed by meiosis of zygote nuclei within the developing asci. A mature ascus contains eight ascospores, all of them haploid. Considered in adjacent twos, starting at one end of an ascus, the ascospores are identical twin representatives of the

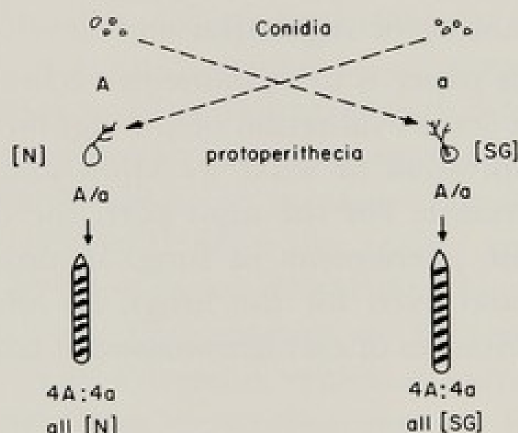


FIG. 1. The basis for a reciprocal cross in *Neurospora crassa*. Typical results are shown for chromosomal heredity involving the mating type alleles *A* and *a*, and for extrachromosomal heredity involving the SG property and its normal (N) alternative.

four products of meiosis. When a cross is made between parent strains having different members in a chromosomal allelic pair, four of the spores from a single ascus represent one genotype and on germination produce cultures of corresponding phenotype; the other four spores are of the alternative genotype and produce cultures phenotypically referable to that genotype. Inspection of Fig. 1 reveals that a chromosomally determined difference should show identical inheritance patterns in the progeny of either member of the reciprocal cross pair. In crosses with normal, SG does not fulfill such an expectation. The results are

SG (protoperithecial parent) × N (conidia) gives all SG progeny
N (protoperithecial parent) × SG (conidia) gives all N progeny

For a given cross, not only do SG and its normal alternative fail to give the expected 1:1 ratio, but they fail to segregate at all. In addition, the

reciprocal crosses do not show identical results. The inheritance pattern may be described as maternal because the conidia are analogous to male gametes and the protoperithecia are analogous to female reproductive organs. The size differential between conidia and the hyphae within a protoperithecium suggests that the maternal parent provides the bulk of cytoplasm for a new generation. Therefore, the fact of maternal inheritance suggests, although it does not prove, that the SG variant depends upon a cytoplasmic difference.

An important consideration in the evaluation of results is that the same crosses that give nonsegregation and maternal inheritance for SG and its N alternative give normal segregations from chromosomal allelic pairs. Figure 2 shows small samples of populations derived from a pair of reciprocal crosses involving SG and N, and also a chromosomal gene pair with members that alternatively determine colonies of large or small diameter. The progeny of either cross shows large- and small-diameter colonies segregating in an approximation of the 1:1 ratio expected when progeny ascospores are collected at random. Neither cross shows segregation for SG and N; in each instance, the phenotype of the progeny resembles that of the maternal parent. Similar results have been obtained with the same reciprocal cross pair when the eight spores of individual asci are taken out separately and then germinated. By this procedure all the products of a single meiotic event may be analyzed. The normal segregation of known chromosomal genes in progenies where SG and N fail to segregate is evidence that the meiotic mechanism is functioning normally in these crosses. The failure of SG to segregate cannot be accounted for on the basis of meiotic anomalies or of differential loss of meiotic products. Furthermore, in a series of comparable crosses involving SG and N, the parental cultures have been marked with chromosomal genes belonging to each of the seven linkage groups of *Neurospora*. The results are consistent with those already described and it may be said that, at least by ordinary techniques, SG is unmappable. In addition, cytological studies have revealed no differences in the chromosomal complement of SG and N cultures.

Other studies have shown that SG is a highly persistent property indifferent to nuclear substitution. By long sequences of backcross in which SG cultures are used as maternal parents and N cultures of *Neurospora* species other than *N. crassa* are used as recurrent paternal parents, the nuclear genetic material originally associated with SG has been replaced with that of totally different cultures. For example, by this procedure SG has been introduced into *N. sitophila,* where its phenotypic attributes and

characteristics of transmission entirely resemble those in *N. crassa*. Even more striking are the results obtained from the introduction of SG into *N. tetrasperma,* a pseudohomothallic species with normally heterokaryotic ascospores that produce cultures in which self-mating occurs. In this species exceptional homokaryotic spores are occasionally formed, and

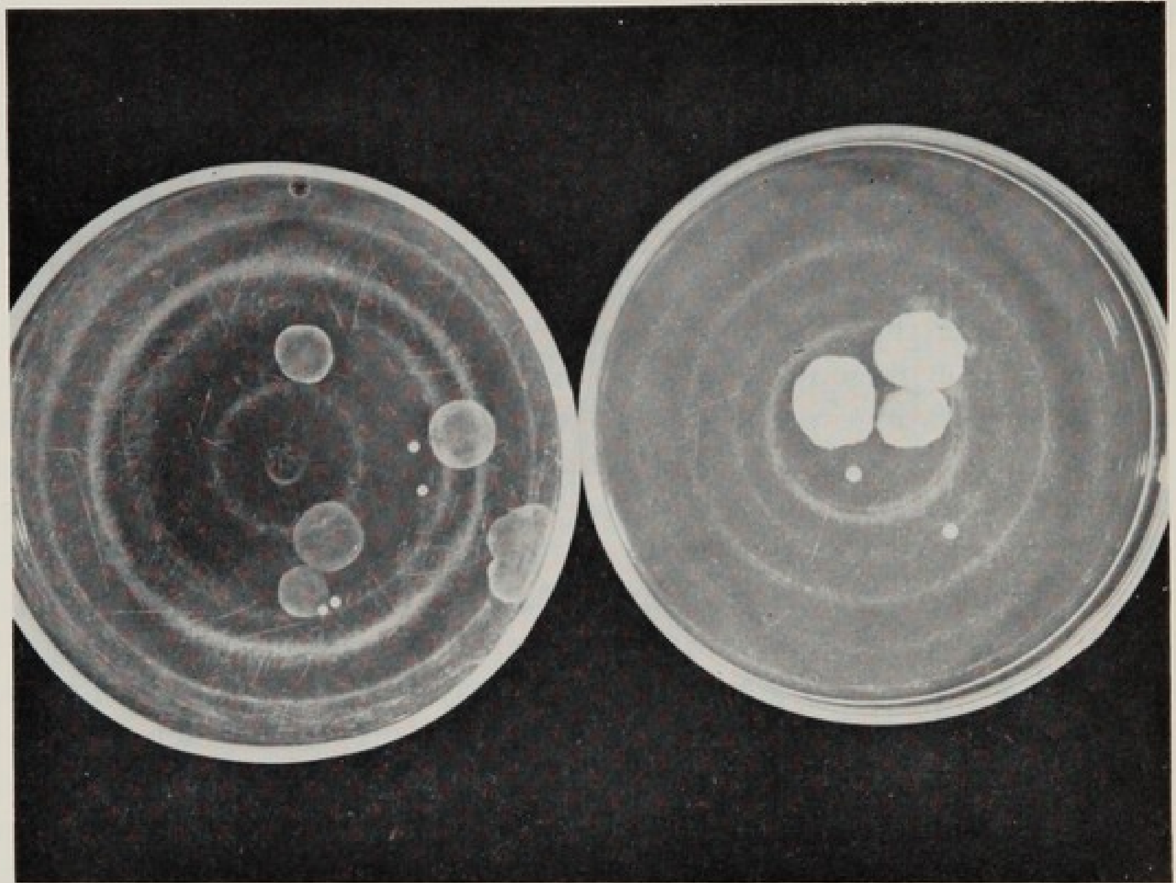


FIG. 2. The experimental detection of extrachromosomal heredity following a reciprocal cross in *Neurospora* in which one parent was SG and the other N. The plate shown at the left was seeded with a small number of ascospores taken as a random sample of progeny of the cross in which SG was maternal parent. All of the colonies are poorly developed because of initially slow growth. The plate shown at the right was seeded at the same time with a sample of spores derived from the mating in which N was maternal parent. All of the colonies are well devoloped because of rapid germination and initially normal growth. Both populations show a 1:1 segregation for an allelic gene pair that determines large- or small-colony diameter.

they give rise to cultures that are either mating type *A* or *a*. Such cultures may be used in controlled reciprocal crosses in the same manner as *N. crassa* or *N. sitophila*.

Starting with a reciprocal interspecific cross between an exceptional homokaryotic culture of *N. tetrasperma* and a culture of *N. sitophila* into

which SG had been introduced by backcross, a further series of backcrosses was utilized to replace the *sitophila* nucleus associated with SG by the nucleus of *tetrasperma*. After 10 generations of backcrossing in which normal *N. tetrasperma* was used as recurrent paternal parent, standard heterokaryotic spores of the *tetrasperma* type were picked and

TABLE I

TRANSFER OF SG CYTOPLASM INTO *Neurospora tetrasperma*[a]

A. Backcross sequence

Backcross generation	Reciprocal cross progenies: SG from previous backcross generation used as ♀		Reciprocal cross progenies: Recurrent parent (*N. tetrasperma*) used as ♀	
	SG	N	SG	N
4	27	0	0	42
6	164	0	0	150
8	449	0	0	392
10	767	0	0	1112
12	1488	0	0	936

B. Self-crossing sequence

Self-generation (starting with ascospores from backcross 12)	Progeny	
	SG	N
1	97	0
2	298	0
3	100	0
4	1823	0
6	1106	0
8	568	0

[a] The maternally inherited character SG arose in *N. crassa* and then was transferred by backcross to *N. sitophila* (Srb, 1958). A. Partial results of a further backcross sequence by which SG was transferred to *N. tetrasperma* which in each case was used as recurrent conidial parent. In each generation a reciprocal cross was made as a test for maternal inheritance. B. Results from a series of self-crosses within *N. tetrasperma* after the nucleus of this species had been combined with SG cytoplasm by 12 generations of backcross. The meaning of the symbols is as follows: SG, variant phenotype; N, normal phenotype; ♀, protoperithecial, or maternal, parent.

the cultures derived from them allowed to self-mate. Further spore isolations and self-matings carried out for a series of generations of sexual reproduction in *N. testrasperma* gave rise only to SG cultures. The results, summarized briefly in Table I, provide convincing evidence for the persistence of SG and for its failure to show segregation correlated with

meiosis. In short, SG was transferred from *N. crassa* to *N. sitophila* and then to *N. tetrasperma,* at least the last mentioned being quite a different species and genetically well isolated from *N. crassa.* The results of a large number of self-matings, with all the segregations attendant on meiosis, were then observed in *N. tetrasperma.* None of this extensive replacement and manipulation of chromosomal genetic material affected the original attributes of SG.

Finally, SG or its normal alternative may be transmitted by purely vegetative means (Infanger and Srb, 1965). It is well-known that in *Neurospora* and other fungi, anastamoses can occur even between cells of identical mating type with the consequence that certain cells may acquire nuclei of different origin and maintain themselves and their vegetative derivatives in a heterokaryotic state (Beadle and Coonradt, 1944). The same fusion process that gives rise to heterokaryosis provides the opportunity for cytoplasmic mixture. If a hyphal fragment of an SG strain carrying a chromosomal marker is manipulated near a fragment from an N strain carrying an alternative chromosomal marker, and hyphal fusion occurs, homokaryotic cultures may subsequently be isolated that show recombinations as well as the original combinations of chromosomal and extrachromosomal properties. The experimental situation is summarized in Fig. 3. In addition, after cellular fusion, a new and ultimately stable phenotype arises, and may be found with either of the two kinds of marked nuclei. The new phenotype, like SG, shows persistent maternal transmission. For the most part, in cultures derived from the fusions of hyphal fragments the two original extrachromosomal properties are mutually exclusive although a single mycelium can yield both SG and N cultures with either of the nuclear markers. The main point, however, is that without any fusion or segregation of chromosomal material, SG may become associated with and maintain its identity in the presence of nuclei of a kind originally present only in N cultures.

In summary, the argument that SG is an extrachromosomal property is based on extensive genetic tests that fail to show any correlated relationship between the transmission of SG and the transmission of chromosomal material: neither SG nor its normal alternative segregates at meiosis; the SG property is unmappable and is indifferent to nuclear substitution; SG may be transmitted vegetatively and maintained without an accompanying transmission of the nuclei with which it has been associated. In contrast, the most apparent positive correlate of transmission of the SG property is cytoplasm rather than nucleus.

The experimental observations that have led to the conclusion that SG provides an instance of extrachromosomal heredity are not unique. They typify a kind of approach and a kind of result that have led to similar conclusions with reference to the heredity of a number of particular characteristics in several genera of fungi. Details of the results of analo-

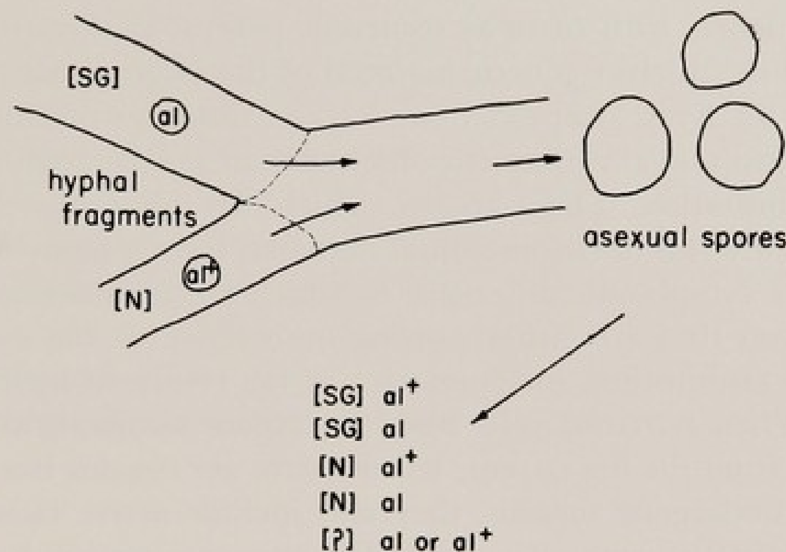


FIG. 3. The vegetative transmission of extrachromosomal properties. Following asexual hyphal fusions extrachromosomal attributes may be found to be recombined with nuclei of a genotype with which they were not previously associated. The extrachromosomal properties are designated by symbols in brackets. The chromosomal gene markers are designated in the standard way; *al* signifies the gene for *albino* and al^+ signifies the allele for normal pigmentation.

gous studies, of course, differ. Some of the differences perhaps reflect little more than variations in the type of experimental manipulation possible for a given species.

In *Saccharomyces cerevesiae,* for example, there is no basis for a reciprocal cross. Fertilization takes place when two cells of opposite mating type simply fuse to form a zygote. In this process, a mechanism for partitioning of the cytoplasm, which apparently occurs when reciprocal crosses are made in *Neurospora,* is not available. Nevertheless, extrachromosomal heredity may be demonstrated on grounds essentially similar to those we have just reviewed. When certain kinds of slow-growing cytochrome-aberrant yeast (called *little* yeast) are crossed with normal, the diploid cells are normal and their meiotic derivatives, the ascospores, also produce only normal cultures (Ephrussi, 1953). Mendelian heredity might easily account for the normal zygotes on an assumption of dominance, but

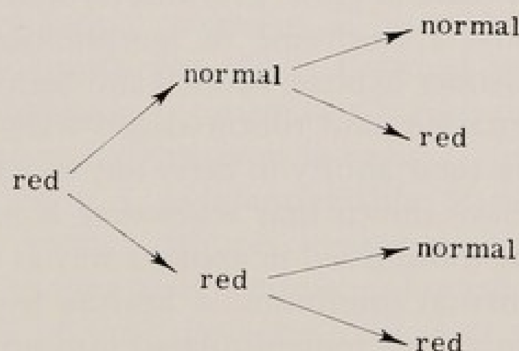
failure of the *little* characteristic to appear among the haploid meiotic segregants is puzzling. If *little* and its alternative, *big*, were dependent on members of an allelic pair, *big* and *little* would be equally represented among the ascospore progeny. By using standard chromosomal markers, Ephrussi has been able to eliminate the possibility that meiosis is abnormal following a *big* × *little* mating. Moreover, he has carried out a backcross sequence, with *little* as recurrent parent, that must essentially have replaced the nuclear genetic material of the original *big* parent with that of the *little*. Even after extensive backcrossing the variant characteristic fails to reappear at a frequency higher than can be accounted for by spontaneous mutation. These results, which are not comprehensible in straightforward terms of chromosomal heredity, have a ready explanation if we assume a cytoplasmic difference in which *big* includes active factors in its cytoplasm that are either missing or inactive in the cytoplasm of *little*. On this assumption, interpretation of the results of hybridization is as follows: When parental cells fuse, the zygote automatically includes active factors from the *big* parent; when spores are cut out from a diploid cell that has undergone meiosis, they also include active factors derived from their originally common cytoplasm. A necessary concomitant assumption is that the cytoplasmic factors either replicate or are somehow reproduced.

Another kind of *little* yeast, called *suppressive*, also gives non-Mendelian results when crossed with *big* (Ephrussi *et al.*, 1955). The diploid cells produced by fertilization, when cultured vegetatively, may give rise either to *big* or *little* clones. Immediate sporulation of presumptive *little* zygotes gives asci with four spores producing varying numbers (zero to four) of *big* or *little* clones. Interpretation of these results is admittedly difficult, but they clearly do not conform to expectations based on chromosomal heredity. It should be noted that previous to this point our examples of extrachromosomal heredity have been characterized by absence of segregation at meiosis. The *suppressive little* attribute appears under some circumstances to segregate, but the segregation is not meaningfully correlated with the distribution of chromosomal material.

As a matter of fact, among instances of extrachromosomal heredity in fungi, systems showing irregular segregation at meiosis are about as frequent as those showing absence of segregation. In *Aspergillus glaucus*, a homothallic species unsuitable for the making of reciprocal crosses, Sharpe (1958) has described a morphological variant that when mated with its alternative normal type gives ascospore progenies that deviate

from the expected 1:1 ratio in varying degrees. In the same progenies, standard chromosomal markers segregate normally. Other instances of apparently the same phenomenon are available (see Jinks, 1964).

Finally, it should be emphasized that evidence for extrachromosomal heredity may be obtained in the absence of sexual reproduction and meiotic segregation. The vegetative transmission of SG in heterokaryons already described provides one example. The *mycelial* variant of *Aspergillus nidulans* is also vegetatively transmissible in heterokaryons, but with the attendant complication of unidirectional transmission from mycelial to normal (Roper, 1958). Similarly, the *red* variant of *A. nidulans* (Arlett *et al.*, 1962) in heterokaryons with its normal alternative behaves in ways that suggest an extrachromosomal system. Even more strikingly, the *red* variant segregates persistently through extended cycles of vegetative reproduction in which the asexual progenies are initiated with single uninucleate spores. The scheme below, if imagined as indefinitely extended, presents the idea of the persistently segregating variant.



Reds and normals occur with varying frequencies as segregants. Occasionally, a normal segregant fails to give *reds* on further vegetative reproduction, but *reds* always give rise to at least a small frequency of normals. Both genetic tests and cytological observation have been used to confirm that the asexual spores used to initiate each cycle of vegetative reproduction are in fact uninucleate. With this assurance, it becomes obvious that what we know of chromosomal genetics is not sufficient to explain persistent segregation. Any hypothesis based on mutable genes, cyclic chromosomal aberration, or unstable aneuploidy requires too many special and implausible assumptions to be convincing. The interpretation made (Jinks, 1964) is that the cells showing segregation are heteroplasmons for two kinds of homologous cytoplasmic determinant. It is supposed that more than one of each kind of homologue is present in cells that give

vegetative segregants, and further, that the distribution of the two kinds of homologous determinants is irregular at the time of cell division. In the particular instance under consideration, it must be supposed in addition that a *red* homoplasmon is lethal, since *reds* that fail to show further segregation have not been isolated. The observation that occasional normal segregants may in a later cycle produce all normals and no *reds* presumably means that such normals are homoplasmons derived from spores in which no *red* determinants were included. Persistent segregation, whatever its interpretation, appears to be of widespread occurrence in fungi and has been reported in several genera.

Fungi also provide an impressive number of instances of more or less persistent phenotypic alteration through an infectionlike process unaccompanied by nuclear migration. A typical instance emerges from the carefully studied phenomenon of senescence in *Podospora anserina* (Rizet, 1957; Marcou, 1961). Strains of *Podospora* cannot be maintained by indefinite vegetative multiplication. After varying lengths of time clones degenerate and finally cease to grow. Previous to total cessation of growth, *senescent* mycelia show a syndrome of morphological anomalies that includes abnormally slender hyphae, swollen and highly vacuolized hyphal tips, increased pigmentation, and ruptured cell walls. *Senescent* but still growing mycelia retain their ability to carry out sexual reproduction, and genetic experiments have shown that *senescence* is maternally inherited. *Senescence* may also be transmitted in another way as demonstrated under the following experimental conditions. A healthy hypha and a fragment from a *senescent* strain are micromanipulated into proximity in a drop of nutrient medium. Characteristically, anastamosis occurs. After several hours, microsurgery is used to separate the hyphae at the point of anastamosis, and they are cultured separately. The mycelium developing from the *senescent* hyphal fragment remains *senescent;* the mycelium developing from the normal hypha invariably shows reduced longevity in comparison with the appropriate controls, and very frequently the reduction in longevity is marked. The use of different genic markers in the normal and in the *senescent* hyphae allowed to anastamose has permitted demonstration that nuclear migration does not occur and therefore cannot account for the transfer of *senescence.* The results of the experiments are therefore consistent with an interpretation that *senescence* derives from a cytoplasmic determinant.

The experimental observations discussed in the preceding paragraphs are representative of the primary evidence for extrachromosomal heredity. We will observe later that other kinds of supporting evidence are also

available. At least, there are other kinds of observation highly consistent with the existence of extrachromosomal hereditary systems. The primary evidence, however, is based on inheritance studies of the kind described; the inheritance in some instances is based on asexual reproduction and in others on sexual reproduction.

The Origins of Extrachromosomal Variability

In no case is the mechanism of origin of extrachromosomal variability clear. Extrachromosomal variants, however, often seem to arise as sudden, heritable, persistent changes, and in the general sense are like mutations. Moreover, the general terminology applied to gene mutations appears to fit the occurrence of extrachromosomal variability fairly well. Mutations from *big* to *little* yeast occur *spontaneously* and can also be *induced* by various agents including acridine dyes and ultraviolet light. By the standards of gene mutation, the spontaneous frequency of occurrence of *little* is high; about 1 out of 500 buds formed by *big* cells gives rise to a *little* variant that shows a pattern of extrachromosomal inheritance. The induced rate with acridine treatment is astonishingly high, and with favorable conditions approaches 100%. Ephrussi and Hottinguer (1950) have provided convincing experimental evidence that acridines actually induce rather than select *little* variants in yeast.

The mycelial variant of *A. nidulans* and the SG variant of *Neurospora* were isolated from normal cultures treated with acriflavine. The red variant of *A. nidulans* arose after treatment of normal cultures with ultraviolet light. In none of these instances is it clear whether the effect of treatment was induction or selection of the variants. The *alba* variant of *A. nidulans,* which does not show Mendelian patterns of inheritance, arises as a frequent spontaneous event. The frequencies of occurrence of *alba* differ from strain to strain and are under the control of a nuclear gene that has been identified in several allelic forms (Mahoney and Wilkie, 1962).

Several of the variations of phenotype implicated with extrachromosomal heredity appear to be recurrent events in the life history of the organism. Senescence in *Podospora* is a case in point. In the Fungi Imperfecti the typical initial morphological attributes of a colony are often superseded by particular variant attributes that appear as sectors in the mycelium. Both in *Pestalozzia* (Chevaugeon and Lefort, 1960) and in *Curvularia* (Cuzin, 1961), the phenotypic attributes of the variant phenotype can be transmitted to mycelia of the juvenile form by contagion. In other words, hyphal anastamosis without nuclear migration is sufficient

for transmission of the attribute. In these instances the occurrence of variation appears to be analogous to a differentiation process since the alterations of phenotype are recurrent and sequential in the life cycle.

On the whole, observations on the occurrence of extrachromosomal variants have not been particularly revealing. At least, the bulk of observations indicate little about the mechanism by which variation is produced. In the one instance, however, in which the origin of variation has been analyzed in great detail, i.e., the origin of *little* yeast, a picture emerges that makes most sense in terms of a cytoplasmic hereditary system. The striking feature of the occurrence of *little* yeast is the high frequency, a frequency never observed for straightforward instances of gene mutation. Ephrussi (1953), in considering this phenomenon, has taken into account both the inheritance pattern of the *little* attribute and the nature of the process by which yeast reproduces asexually. The inheritance pattern, already described, is non-Mendelian; the asexual reproduction of yeast is by budding. It is easy to visualize spontaneous mutations to *little* simply as failure of a particular kind of cytoplasmic hereditary determinant to be included in a bud before it is compartmented off from the mother cell. Such a hypothesis is most plausible if the cytoplasmic determinants are visualized as particulate, self-replicating, and relatively small in number. The enormous efficiency of acriflavine in inducing *little* yeast can be interpreted on the basis of inhibition or alteration of replication of the cytoplasmic particles. This interpretation gains plausibility from the experimental finding that treatment with acriflavine is effective only on growing cultures. In summary, both the exotic inheritance pattern for *little* yeast and an equally unusual mutational pattern can be comfortably accommodated within an interpretational scheme based on cytoplasmic particles of hereditary significance. A scheme of the same kind is able to accommodate observations on the characteristics of mutation and transmission that have been observed for the *alba* variant of *Aspergillus* (Wilkie, 1964), and has application elsewhere as well.

Phenotypic Effects

The range of phenotypes involved in extrachromosomal transmission is diverse. Many of the heritable variants have been recognized as alterations of morphology or of color, and very often a given variant is characterized by a syndrome of visibly deviant attributes. In a number of instances extrachromosomal variants are distinguishable by their abnormally slow growth or deferred germination of spores. The attributes affected by extrachromosomal heredity are by no means trivial; they include such charac-

teristics of biological significance as longevity, ability to form asexual spores, and ability to carry out sexual reproduction. Considered as a whole, these characteristics have not been susceptible to the kind of biochemical analysis so readily available for the study of auxotrophic gene mutants and their normal alternatives. Certainly one of the unsatisfactory aspects of our knowledge of extrachromosomal heredity is that very few cases have as yet provided a basis for understanding in terms of biochemistry and physiology.

It remains an open question why the phenotypes of extrachromosomal variants in the fungi have not included simple growth factor requirements and other expressions of biochemical lesion commonly found in microorganisms as a result of chromosomal gene mutation. That they may eventually be found is suggested by the existence of such extrachromosomal variants as acetate requirement and streptomycin resistance in *Chlamydomonas* (Sager and Ramanis, 1963). The failure to find extrachromosomal variants of this kind in fungi may be no more than a flaw in the processes by which variants have been identified and selected. On the other hand, reasons why such variants cannot exist can be imagined.

Fortunately, a few of the phenotypes involved in extrachromosomal heredity have been analyzed in ways that give insight into function at the biochemical level. *Little* yeast, in comparison with its *big* alternative, is lacking in cytochromes a and b, and shows other aberrations of the cytochromes. Certain enzymes that function in respiratory metabolism, for example cytochrome oxidase and succinic dehydrogenase, are extremely deficient. One can scarcely ignore the fact that the seat of biochemical functions with which the *little* mutation interferes is a cytoplasmic organelle, the mitochondrion. Studies in ultrastructure further implicate this organelle in the phenomena presented by *little* yeast. Yotsuyanagi (1962) has shown by means of electron microscopy that the mitochondria of *little* yeast are abnormally structured.

The general features of *little* yeast are paralleled by those of *poky Neurospora* (Mitchell and Mitchell, 1952). *Poky* is a slow but irregularly growing strain with deviant attributes that are maternally inherited. Like *little* yeast, *poky* is characterized by aberrations of the cytochrome system (Mitchell *et al.*, 1953) and defective mitochondria.

Interactions

In the gross sense, the typical manifestations of interaction between chromosomal mutants are found also to result from interactions between extrachromosomal variants or between extrachromosomal variants and

chromosomal mutants. For the purposes of this paper, the chief interest in these interactions lies in the fact that they exist. They will therefore be dealt with only briefly as significant biological phenomena for which the mechanism remains virtually unknown. It should be remembered, however, that only quite recently has a substantial basis emerged for interpreting such interactions as suppression and complementation as they exist in purely Mendelian systems.

Complementation

Pittenger (1956), using standard techniques for the forcing of heterokaryosis in *Neurospora,* has been able to examine the effects of combining two different extrachromosomal variants vegetatively. One of these, *poky,* has already been described; the other, *mi-4,* is also a slow grower that can be shown to have an aberrant complement of cytochromes. The result of the combination of the two variants was a mycelium that for an extended period of time grew at essentially the same rate as normal controls. The experiment was conducted in a way that made it possible to exclude the theory that the heterotic effect might be due to interaction directly referable to the nuclear components of the mycelium. Therefore, the rapidly growing mycelium derived from the combination of the two variants appears to have been a complementing heteroplasmon. Although growth of the heteroplasmon was normal, the cytochromes were not. Moreover, the heteroplasmon did not maintain indefinitely, but eventually segregated into its component types. These observations signify that the initially normal growth of the heteroplasmon was not due to some recombination process that produced a normal set of cytoplasmic factors. Instead, normalcy for growth was due to complementation at the phenotypic level. In any case, it must be inferred that in the functional sense *poky* and *mi-4* represent nonidentical lesions.

Suppression of Mutant Phenotype

Mutation at a chromosomal locus in *N. crassa* gives rise to an allele that acts as a phenotypic suppressor on *poky* (Mitchell and Mitchell, 1956). In the presence of allele *f, poky* strains grow at nearly the wild-type rate. The cytochromes, however, remain aberrant. If a suppressed *poky* strain is used as maternal parent in a cross with f^+, half the progeny are of standard *poky* phenotype. This result indicates that allele *f* does not impose a persistent heritable change on the cytoplasmic factors that account for the *poky* phenotype. It is noteworthy that in regard to interaction phenomena such as complementation and suppression, systems

involving extrachromosomal heredity resemble classic Mendelian systems in that interaction is clearly at the phenotypic rather than the genotypic level.

Discussion

A description of extrachromosomal heredity based on observations in the fungi does not present quite so diverse a picture as if consideration were also given to comparable observations in protozoa, bacteria, and green plants. Nevertheless, the picture presented by fungi can be considered reasonably typical. It is based on the same kind of approach utilized for the study of extrachromosomal heredity in other groups of organisms; the gaps are similar and so are the difficulties of interpretation. The same basic issues arise either with a plastid or a mitochondrion when we contemplate the evidence that organelles outside the nucleus may have genetic continuity and bear genetic information.

First, however, let us consider what we do know as the result of experimentation with extrachromosomal systems. It seems to the author that the question of the reality of extrachromosomal heredity is no longer a matter for debate among biologists. Adequate studies on a variety of organisms demonstrate clearly the existence of persistent non-Mendelian patterns of inheritance. Some of these patterns are segregational, showing that the systems have flexibility as well as persistence. Although certain observations indicate no more than the fact that the inheritance pattern is not correlated with the distribution of chromosomal material, others point directly to some component of the cytoplasm as a determinant of the pattern of inheritance observed. In a few of these instances, mitochondria are certainly implicated, but whether they are primarily determinative remains questionable.

Extrachromosomal hereditary systems undoubtedly carry information of some sort. Several studies reviewed earlier in this paper demonstrate that persistent heritable properties may be transmitted in the absence of transmission of nuclei. When the transmission of these same properties is examined in relation to the standard processes of sexual and asexual reproduction these properties show extrachromosomal patterns of inheritance.

Extrachromosomal heredity can no longer be considered inconsequential. The number of known cases in fungi is now quite large and is distributed among most of the genera given serious genetic study. Moreover, the biological attributes involved are significant, and extrachromosomal hereditary variables may have a profound influence on the function

and survival of the organism. In the Fungi Imperfecti, systems having properties of extrachromosomal heredity are recurrently involved in the regular developmental sequence for the life history of a culture.

Variability in extrachromosomal systems may arise as sudden, persistent, heritable changes analogous to mutations. These changes must have adaptive importance; they should be subject to selection; and, therefore, they would seem to be inevitable participants in evolutionary processes.

What significant matters of principle remain unknown for extrachromosomal systems? For the most part they have to do with mechanism and can be brought into focus by a single question: Is extrachromosomal heredity based on primary sources of genetic information that exist in the cell but outside the chromosomes? In other words, are there entities outside the chromosomes that have the basic attributes of genes? A description of these attributes may be phrased roughly as primary information that provides for both accurate replication of the entity and ultimate definition of the potential for carrying out a particular heterocatalytic process.

The questions just posed are not the only ones that can, or should, be asked about extrachromosomal heredity. They point to a basic issue, however, that arises naturally in the context of present knowledge and the means by which it has been obtained. Present knowledge tells us that all heritable attributes have much in common, but that they may nevertheless be placed into one of two sets. Into whichever set they fall, heritable attributes are similar in that their transmissibility under ordinary circumstances depends upon cellular continuity and that they show great persistence occasionally interrupted by the kind of sudden, heritable change that fulfills the general criteria for mutability. The sets differ in that in one, the pattern of appearance of phenotypic attributes through a sequence of cell generations is directly referable to the details of transmission of chromosomal material. In the other, the pattern of transmission of phenotypic attributes is not referable to chromosomal transmission and often, if not always, is directly correlated with cytoplasmic as distinct from nuclear continuity. Are these observations sufficient to establish the existence of two systems of heredity, admittedly interrelated, and highly similar except that one depends on genes in chromosomes and the other on gene-like entities outside the chromosomes? The observations in themselves are not sufficient. The category designated extrachromosomal heredity has been established largely through observations of phenomena not in conformity with traditional patterns of chromosomal heredity readily recognizable by a fairly simple relationship between genotype and

phenotype. At least since the proposal of Delbrück's (1949) model for alternative cytoplasmic metabolic states without nuclear genetic change, students of extrachromosomal heredity have been aware that non-Mendelian inheritance patterns, even when of great persistence, need not signify a source of primary genetic information other than in chromosomes. With the discovery of operons and other systems for controlling gene action, one can devise models, based on demonstrable phenomena of chromosomal genetics, that are formally sufficient to account for many of the instances of extrachromosomal heredity. It must be said, however, that in no instance has such a model yet been shown to be applicable. Counter models based on the assumption of genetic entities in the cytoplasm may in particular instances have equal or greater plausibility. Thus, two contemporary books on extrachromosomal heredity (Jinks, 1964; Wilkie, 1964) can give reasonable treatment to the same experimental field from rather different interpretational viewpoints. Elegant and extensive experiments on a given system, for example the barrage phenomenon in *Podospora* (Rizet and Schecroun, 1959; Beisson-Schecroun, 1962), are still interpretable either on the basis of rather strict chromosomal control or in terms of partial cytoplasmic genetic autonomy.

Since no definitive interpretation of mechanism is at hand, the relative plausibility of different models in reference to particular systems of extrachromosomal heredity will not be considered here. Since the exciting possibility also remains that primary genetic systems may exist in the cytoplasm or elsewhere outside the chromosomes, it may be worthwhile in conclusion to consider what would be necessary for the proof of such a system and how such proof might be established.

Certain of the segregational phenomena and also some of the mutation-like phenomena in extrachromosomal systems are most readily visualized as having a particulate basis in the cytoplasm. Particles can be conceived in various ways and as having various properties (Catcheside, 1956), but the cytoplasm of fungi includes at least one kind of known particle that has been clearly implicated in two instances of extrachromosomal heredity—the mitochondrion in *little* yeast and *poky Neurospora*. Recent studies by Luck and Reich (1964) demonstrate that the mitochondria of *Neurospora* contain DNA. Therefore, a cytoplasmic particle contains a substance the properties of which determine the replicative, heterocatalytic, and mutational attributes of chromosomal genetic systems. The mere presence of DNA in a cytoplasmic particle, however, does not prove the existence of a primary genetic function for that particle; it only lets us visualize in familiar terms one of the ways in which a genetic particle

in the cytoplasm might operate. If a specific base composition for mitochondrial DNA were found to be maternally inherited or transmissible by cytoplasm in the absence of nuclear transmission, and if it were found also to account for the specificity of a particular protein, then the existence of a very gene-like entity in the cytoplasm would be definitively established. Nevertheless, the recognition of one such instance would not mean that all instances of extrachromosomal heredity have a similar basis. It would not be surprising if different mechanisms, including some that are episomal, are found to be responsible for different instances of cytoplasmic heredity.

An additional experimental finding should be mentioned before the close of this discussion. The *little* phenotype in yeast can arise as the result of gene mutation and be transmitted in the usual fashion of chromosomal heredity (Ephrussi, 1953). Mitochondria, then, are certainly not autonomous genetic particles. Even genes can be the receptors as well as the transmitters of information. That any component within the cellular system will be found to have true autonomy in its various functions is quite unlikely. The different means for providing continuity in biological reproduction, when reasonably well understood, will certainly show a high degree of integration and interrelation.

Acknowledgments

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Final Remarks: "Why So Much DNA?"

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Now that all the papers on Reproduction of Subcellular Systems have been presented I am convinced, and perhaps you are too, that hereditary material is localized and generated not only in the nucleus but also in the cytoplasm. The conclusion that hereditary factors are present in the cytoplasm has been a long time in coming—since 1906 in fact, when Correns first suspected the phenomenon of cytoplasmic inheritance. The recent crucial finding that these hereditary cytoplasmic factors are made of DNA has made cytoplasmic inheritance acceptable to the most critical of geneticists.

This year, as you know, is the hundredth anniversary of Mendel's discovery of unit factors of inheritance. It is fitting that the conclusion to 100 years of study should be the very satisfying and unifying concept that the hereditary factors in both the nucleus and cytoplasm of higher cells are composed of DNA.

Now, I would like to present very briefly a problem that has arisen in my mind concerning the significance and activity of the DNA in higher plants and animals. I think the problem arises from the fact that genetics has used Mendelian analysis almost exclusively. You will recall that the success and power of Mendelian analysis of unit factors of inheritance rests on two remarkable properties. First, all other factors in the entire cell may be kept constant only a single genic molecule changed. Second, the change in this single genic molecule can be recognized because the molecule is a template which becomes magnified by translation into a single type of enzyme yielding several million molecules as products; this is called the phenotypic expression of the gene. Mendelian analysis is suitable for one or two alleles of a gene, but when there are many more than two alleles, Mendelian analysis becomes very cumbersome or even impossible. This is true not only for DNA of the cytoplasm but also for DNA of the nucleus. For example, a liver cell contains about 1000 mitochondria; each mitochondrion may contain one double-stranded multigenic DNA chromosome. Suppose a muta-

tion occurs in a gene in one of the mitochondria of a cell. How do we analyze for such a mutation in this single mitochondrion among a thousand other mitochondria? Similarly, if there were a large number of duplicates of a gene in nuclear DNA we would be unable to recognize a mutation in one of these genes. Worse than that, we would not be able to recognize the existence of the gene.

How many genes have we been unable to recognize because they do not lend themselves to Mendelian analysis? Is there much DNA in the nucleus, the significance and activity of which we do not know? I think there is.

A few years ago I happened to look up the amount of DNA present in a bacterial cell, in a higher plant cell, and in a higher animal cell. Both the plant cell and the animal cell have about 1000 times more DNA than does the bacterial cell, and I was greatly perturbed by this. Last year Barry Commoner assembled DNA data from various cells, and from his data a remarkable curve may be constructed. The curve shows a direct relation between the protoplasmic volume of a cell and its DNA content. The larger the cell, the more DNA—and this relation is valid for five orders of magnitude. The amount of DNA in a higher plant or animal cell—1000 times more than in a bacterial cell—is essentially the DNA of the nucleus. The cytoplasmic DNA of mitochondria and plastids constitute only about 1% of the total DNA of a cell.

Why does a large cell have more DNA than a bacterial cell? I do not have the answer, only the following impressions.

A bacterium like *E. coli* can make all of its protoplasm from glucose and inorganic salts. It is a remarkably capable biochemist; and can even make amino acids and vitamins that a mammalian cell cannot make. Why is more DNA than this required for a higher plant or animal cell?

Perhaps a large cell has to make more protein molecules of the same kind. It might do this by having more duplicate genes of a particular kind. This possibility is suggested by the anatomy of *Paramecium*. *Paramecium* is a large cell containing much DNA; it has a tiny packet of DNA, the micronucleus, that regulates hereditary details; and for cellular biosynthesis, it has a large macronucleus representing many copies of the micronuclear DNA. This arrangement is logical and sensible.

We do not find this arrangement, however, in the cells of higher plants and animals. For example, in the synthesis of hemoglobin in a mammalian erythrocyte there is no evidence for many duplicates of the genes that code for globin. The evidence clearly indicates that there are only

two functional alleles that code for the α chains and two that code for the β chains of hemoglobin synthesis in the red cell of an adult. Therefore, these four alleles are sufficient to provide the templates for practically all the globin synthesized by the erythroid cell in a period of 24–48 hours, the period of active synthesis of hemoglobin.

Then why is there a large amount of DNA in a mammalian nucleus? Perhaps this quantity of DNA is related to differentiation. Cells of mammals are highly differentiated and much of the DNA may code for differentiation processes. Perhaps the genome consists of many duplicates of genes or operons, some of which are active in one cell type and others in another cell type. We could even go to the extreme of suggesting that every different kind of nerve cell requires a different combination of the multiple genomes available. All of this differentiation might readily account for the fact that a mammalian cell has 1000 times more DNA than a bacterial cell. This sounds reasonable until we consider the higher plant cell. Such a cell is relatively simple in terms of differentiation, and yet it also has 1000 times more DNA than a bacterial cell.

Trying an entirely different approach we might suppose that there are 10 genes for the regulation of 1 gene, and also that there are other genes like heterochromatin which perhaps double the DNA. This would account for a twenty-fold increase over the DNA content of a bacterium, and we would still be fiftyfold away from the DNA of a large plant or animal cell.

Could DNA have a function in addition to that of template? Commoner has suggested this possibility, and has considered that this other function might in some way be related to cell metabolism. This may be true, but I still feel more satisfied thinking that DNA serves primarily as template.

Thus the problem still remains. Why is there so much DNA in a higher plant or animal cell? Is it to synthesize proteins of one kind at a faster rate? Is it for processes of differentiation? Or does DNA have a different role in addition to coding?

It seems most reasonable to me to consider that the high DNA content represents duplicates of genes, operons, etc. If there are many duplicates of a gene we have failed to find them because the methods of Mendelian genetics are not suited to such an analysis. Cytoplasmic inheritance which has been suspected since 1906 was largely ignored because genes present in many duplicates could be analyzed only with great difficulty. Nor do we have adequate means for analyses available today.

It seems to me, on the hundredth anniversary of Mendel, that

Mendelian genetics may have provided us with the ability to analyze perhaps 1% of nuclear genes of the higher plants and animals. We need new methods and ideas to learn how to analyze for the gene functions of the remainder of DNA in the nucleus, as well as in the cytoplasm. Perhaps we need a new Mendel with a computer, or perhaps a method and device by which we can insert a double-stranded helix, obtain base sequences, and have a "magic" analyzer tell us what function they are coding for. Maybe we will have these by the second centenary of Mendel. I hope it will be much earlier.

THE REPRODUCTION OF CELLS

Haploidy versus Diploidy in the Reproduction of Cell Type

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Introduction

Haploid cells and organisms will be the main subject of this paper, and they will be compared to diploids in terms of their capacity for growth and differentiation. Examples of both plant and animal species will be discussed. Some emphasis will be placed on work performed in our laboratory on haploid tissue cultures from plants, particularly those derived from *Ginkgo biloba* L.

If we consider the dominant forms of plant and animal life which survive today, we observe that they are primarily diploid in chromosome complement. That is, they develop and function in a diploid ($2n$) state of complementary hereditary information, and transmit their characters through single genomes which are haploid (n). This simple well-known generalization is based on observations of the many biological forms which confront us in the field and in the laboratory, and this generalization holds, even though there are many interesting and informative exceptions.

Concerning the general framework of biological forms and functions, we may ask: What is special about the diploid state in comparison with the haploid? Are the differences between diploid and haploid cells reflected in their functions? We recognize, for example, that the haploid state represents a type of specialization especially effective for sexual reproduction. Similarly, we know that the diploid state not only assures survival and dominance of the species, but that it is also essential for the organization of complex organisms. In other words, complementary information systems are usually needed for completely normal ontogeny of the organism.

The fusion of haploid nuclei to form the diploid organism requires a periodic reduction division. In a sense, this reduction division may be regarded as a hereditary cloning process because the gametes derived

from reduction division, as well as the zygotes derived from their fusion, are unique creations. Within this context of continuous variation, where sex and modulation of characters are involved, many questions can be asked. For example, were primitive cells haploid? Did the first fusion of haploid nuclei confer a greater capacity for competition and survival? Was sex evolved from some of these early advantageous events? These are all interesting and pertinent queries. Our main concern, however, is with the following questions: What are the relative capabilities of haploid cells and organisms in comparison with those of their diploid counterparts? What is known in this area and what are the basic problems?

For coherence, and in order to limit the scope of reference, it will be useful to define a few terms. A haploid cell or organism, for example, is understood to have a single basic set of chromosomes of a given species. This set may be male (androgenetic) or female (gynogenetic); one genome, one gametic chromosome constitution, and haploid all are synonymous terms conventionally designated as (n). Euploids are multiples of the basic number; hence, diploids are the first of a series that includes triploids and tetraploids; higher genome sets are designated polyploids (e.g., autopolyploids which possess similar genomes, and allopolyploids which have dissimilar genomes).

Characteristics of Haploids

The most abundant haploid organisms are the lower forms, i.e., the algae, bacteria, yeasts, fungi, mosses, and liverworts. Haploid animals include the male bees and wasps, other Hymenoptera, and some members of the Rotifera, Homoptera, and Thysanoptera; haploid males usually develop from the eggs of diploid females, but the production of parthenogenetic haploid females is much less common (Suomalainen, 1950). Among the higher plants, haploidy is known to occur in over 71 species of 39 genera in 16 families (Kimber and Riley, 1963). Haploids are reported in such well-known genera as *Oenothera, Epilobium, Zea, Gossypium, Nicotiana, Datura,* and *Triticum*. Some of them are spontaneous haploids of uncertain origin, and others are the result of hybridization or experimentation. Haploids derived experimentally, of course, provide more information, since their origin can be determined. Most haploids are derived from the cells of the embryo sac, and hence are maternal in origin; androgenetic haploids are relatively rare. The first examples of androgenesis in maize (*Zea mays*) were reported by Goodsell (1961) and Chase (1963). Male sterile monoploid paternal plants were obtained in very low

frequencies (1/80,000) from special crosses. The sperm nucleus from the pollen apparently united with the cytoplasm of the egg which contained the plasmogenes for male sterility. The low frequency of progeny with this character indicates that the method is of little use in breeding programs unless special lines or artificial induction can be used to increase its frequency. The significant fact, however, is that the expression of male sterility is governed by cytoplasmic factors from the maternal side. It will be very interesting to discover the nature of this type of cytoplasmic inheritance, especially to determine whether some particular satellite DNA is involved.

Haploid higher plants which have been found or induced experimentally, generally exhibit a weakened structural constitution. Haploid cotton plants, for example, possess zigzag stems, short internodes, usually produce no pollen, seeds, or bolls, and develop leaves and flowers reduced in size (Meyer and Justus, 1961). Doubling the chromosome number by colchicine treatment, however, may restore vigor to the progeny as shown in Fig. 1; leaves, flowers, and fruit are then restored to normal proportions. The origin of these haploids is not known, i.e., whether they develop parthenogenetically from the unfertilized egg, or androgenetically from the generative nucleus, although the former possibility seems more likely. These plants are propagated vegetatively by cuttings and usually do not produce either functional pollen or viable seeds. Haploid tomato plants of known genotype, however, were propagated vegetatively by Lindstrom (1941) for over 14 years and were found to be quite stable; homozygous diploids derived from these plants were highly fertile. This stability in cotton is an exception, however, because plants with a single genome usually are quite variable in character; genetic balance and phenotypic uniformity are generally not maintained. Induced haploidy often leads to a serious disruption of development, especially in normally outbreeding heterozygous plants. Thus, the structural adaptations to outbreeding which are seen in some higher plants, serve to maintain heterozygosity; circumvention of this by the production of haploids usually leads to genetic imbalance. In contrast, highly inbred species have a minimum of allele differences because the introduction of new alleles is restricted. Haploids derived from inbred species might be expected to develop with greater frequency but with reduced vigor in comparison with haploids from heterozygous species which are fewer and more robust.

Animals of gametic chromosome constitution generally exhibit what is known as a haploid syndrome (Moore, 1955; Briggs and King, 1959; Subtelny, 1958). Enucleated eggs of *Rana pipiens* implanted with nuclei

from haploid androgenetic blastulae develop as far as the beginning of gastrulation, but then become abnormal. Deficiencies in the gut, central nervous system, sense organs, and pronephros and cardiovascular systems, are collectively known as the haploid syndrome. In some of the recipient eggs development is delayed and the chromosome number is doubled. These androgenetic autodiploids develop normally up to the early post

Fig. 1. Leaf and flowering branch from doubled haploid cotton plant (left) derived from haploid plant (right) by colchicine treatment. From Meyer and Justus (1961).

neurula stage and thereafter show abnormalities. The larvae feed and survive, but usually die before metamorphosis. Examples of these homozygous diploids and their haploid and diploid controls are shown in Fig. 2. Gynogenetic diploids are also retarded, but some reached the frog stage (Moore, 1955). Apparently, the maternal genome carries sufficient information and suitable cytoplasm for development to continue through metamorphosis, whereas the growth of androgenetic homozygous diploids is arrested earlier.

More than forty years ago Bridges (1925) described mosaic female

individuals of *Drosophila melanogaster* which contained patches of haploid male tissue. These mosaic fruit flies were unusual because most natural haploids in animals are males. Bridges confirmed the presence of several sex-linked patroclinous characters and the single X chromosome in the haploid male tissue. In contrast, the development of fertilized sub-haploid eggs of *Drosophila* (Briggs and King, 1959) was arrested during cleavage; hyperhaploids of frogs terminated their development during gastrulation, and hyperdiploids of axolotls matured only to the feeding larval stage. Aneuploids in all these cases were sufficiently imbalanced to

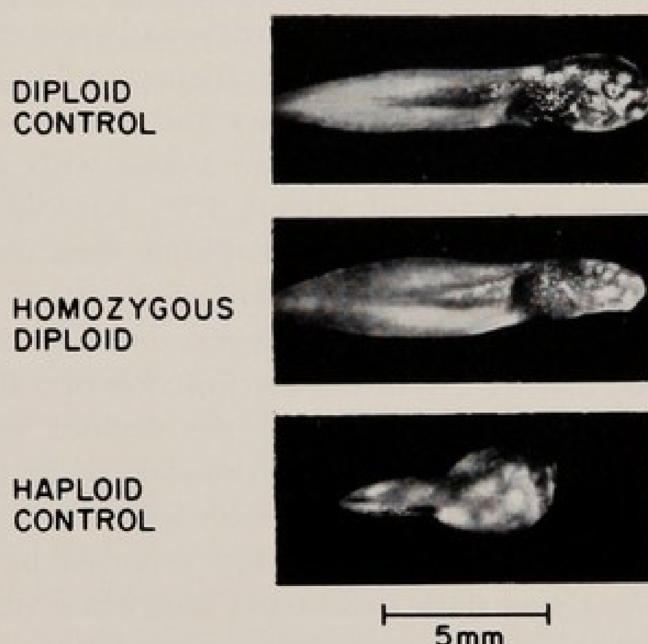

Fig. 2. Androgenetic homozygous larvae of *Rana pipiens* compared to a diploid control and an androgenetic haploid control. From Fig. 6, Subtelny (1958, p. 282).

impair development. We can assume, therefore, that a correct balance of genes in a complementary diploid condition is required for normal ontogeny; more evidence in support of this claim will be presented later.

Induction of Haploidy

The artificial production of haploids may be brought about by a variety of techniques. Ultraviolet treatment of newt sperm, for example, is effective in inducing haploid parthenogenesis from eggs of three species of *Triturus* (Selman, 1958); similar treatment of pollen induces haploid parthenogenesis in tobacco (Ivanov, 1938). Radiation treatment of pollen in general, however, does not enhance haploid frequencies (Brewbaker and Emery, 1962), and the results vary from species to species. In some

Zea mays hybrids there may be an increase in haploids (22/20,869 for irradiated and 1/11,707 for control), but the number of spontaneous haploids in some inbred lines may exceed 1%; in *Antirrhinum majus* maternal haploids may be about 0.05% (Knapp, 1939).

Induction of haploids from higher plants usually occurs through parthenogenetic development of the egg. In special cases, such as in *Melilotus* hybrids (Jaranowski, 1961), the synergids of the embryo sac may be stimulated to develop with the result that one or two synergid-derived haploid embryos and the normal diploid embryo may be found in a single seed. In this instance the haploid embryos compete satisfactorily with the diploid embryo, develop to maturity, and survive.

Other crossing techniques make use of alien cytoplasm to enhance the frequency of haploid development (Kihara and Tsunewaki, 1962). When *Aegilops caudata* cytoplasm was present in a cross with *Triticum vulgare* var. *erythrospermum* (a variety of wheat), 1.7% haploids were obtained; in a cross with Taylor's *Triticale*, 53% haploids were obtained. Without *Aegilops* cytoplasm, no haploids were formed in either cross. These experiments are interesting because they suggest that a suitable composition of cytoplasm in the mature egg favors the function of a single genome. In the absence of these favorable factors, the diploid state is required; perhaps the diploid condition provides a similarly favorable cytoplasm.

A marked difference in the frequency with which haploids occur in the Mexican axolotl (*Siredon mexicanum*) was reported by Humphrey and Fankhauser (1957). Using dark (wild-type) and white (mutant, recessive) breeding stocks, they were able to detect androgenetic and gynogenetic haploids in various crosses by using color, number of chromophores, and the haploid syndrome as distinguishing characters; chromosome counts of tail clippings were also made. In crosses of white females with dark males, 213 offspring were spontaneous gynogenetic white and 11 were androgenetic dark haploids out of 31,273 examined; in the reciprocal cross, 4 gynogenetic and 17 androgenetic haploids were obtained from 23,154 offspring. Judging from the preponderance of female haploids we might conclude that this is probably a genetic character associated with the mutant strain of axolotl. When dark females were mated with white males, however, and the eggs placed in a refrigerator for 9–18 hours, a large number of white male haploids resulted. There was a low survival rate in these experiments; only 1/194 haploids were obtained with 1–9 hours cold treatment; however, 63/110 survivors of an extended cold period were haploid. It was suggested that prolonged cold treatment essentially eliminated the egg nucleus of the dark females from participation in development and

hence increased the incidence of white haploid male axolotls in these crosses.

This temperature-induced haploidy in the axolotl recalls some observations made on pollen development in *Hyacinthus orientalis* by several workers, and reviewed and extended by Naithani (1937) and Stow (1933). Stow found that he could induce embryo sac development in pollen from a particular variety of hyacinth by elevating the temperature in the premeiotic phase. Some of the pollen-derived embryo sacs were penetrated by germinating pollen tubes and the sperm nuclei released into the embryo sacs. This case of temperature-induced sex inversion in pollen was authenticated by Naithani who observed it as a normal occurrence in another variety of the same species.

Radiation Effects

The aging process in living organisms involves many factors, one of which is radiation damage. It might be expected that diploidy would provide an advantage over the haploid state, since each character would have an allele. Evidence to support this view has been obtained from experiments on *Habrobracon* (Clark *et al.,* 1963). Haploid and diploid males having the same life span were X-irradiated at dosages known to decrease the life span. The fact that irradiated diploid males lived longer than haploids, suggests that X-radiation damage (and subsequent aging) is retarded when alleles are present.

Other evidence comes from investigations on higher plants by Sparrow *et al.* (1961) who demonstrated a correlation between nuclear volume and tolerance to chronic radiation: the larger the nuclear volume, the greater the sensitivity to radiation; similarly, the higher the DNA content (at a given genome level), the greater the sensitivity. When replicate genomes were present, however, as in the case of two polyploid series with *Chrysanthemum* and *Sedum,* there was an increased resistance to radiation damage.

Quantitation and Biochemistry

The quantitative relationship between haploid and diploid cells has been determined in a variety of ways and, in general, is of the order of 1:2. An excellent demonstration was presented by Rudkin *et al.* (1955) who determined the DNA content of haploid and diploid salivary gland chromosomes of *Drosophila melanogaster*. They measured the ultraviolet absorption of paired chromosomes and of a single homologous chromo-

some in the same cell of a triploid fruit fly. The extinction values and the chromosome numbers fulfilled the criteria of Lambert's law of proportionality, indicating that the DNA content of paired chromosomes was approximately twice that of the single chromosome.

Other work by Mangelsdorf and Fraps (1931) established a quantitative relationship between the genes for yellow pigmentation (*Y*) and the amount of vitamin A in corn endosperm. This relationship is shown in Table I: The presence of the *y* allele produced small amounts of vitamin A, whereas one, two, and three doses of the *Y* gene produced corresponding increases in vitamin A content.

TABLE I
QUANTITATIVE RELATIONSHIP BETWEEN GENES FOR YELLOW PIGMENT (*Y*) AND VITAMIN A CONTENT OF CORN ENDOSPERM[a]

Number of genes for yellow	Factorial composition of endosperm	Units of vitamin A per gram		
		1928	1929	Average
0	*y y y*	0.05	0.05	0.05
1	*y y Y*	2.50	2.00	2.25
2	*y Y Y*	5.00	5.00	5.00
3	*Y Y Y*	7.00	8.00	7.50

[a] After Mangelsdorf and Fraps (1931).

Another long recognized factor in haploid and diploid development is the nuclear-cytoplasmic ratio. Briggs (1949) demonstrated clearly and effectively that androgenetic haploids of *Rana pipiens* induced from small ova were smaller, more normal, and better able to feed, than haploids from large ova; the latter lasted only 18 days whereas those from small ova lasted 9 months. The functions of the single genome seem to be more dependent on the cytoplasm than those of the diploid genome. The surface-volume relationships of haploid cells appear to be nearer the critical level than those of diploid cells.

In the wasp *Mormoniella,* Mortimer and von Borstel (1963) were able to show that the sensitivity of sperm to radiation (in strains with dominant radiation-induced lethality) was doubled in diploid sperm in comparison with the normal haploids. This observation suggests a strict correspondence between the amount of genetic material and the radiation effect. Other evidence of this relationship was reported by Olsen and Ogg (1963) for haploid and diploid strains of *E. coli:* Diploids were found to be more resistant to X rays than haploids. These findings, however, were

incidental to their investigations. They used formic hydrogenlyase activity to show that haploid and diploid cells respond differently when placed under anaerobic conditions in a growth medium without formate: When subsequently exposed to formate, the haploids produced hydrogen, while the diploids did not. Under inducing conditions (i.e., in a medium containing formate) the induction lag was longer in diploids and the maximum inducible rates were lower than for haploid cells. Thus, on a quantitative basis the cells of *E. coli* and other organisms reflect their haploid-diploid constitution, but on a qualitative basis, the effect is more subtle as shown by the formic hydrogenlyase experiments. The addition of a second genome actually repressed enzyme activity, rate of inducibility, and the rate of hydrogen production from formate. Other kinds of measurements on polyploid series in yeasts (Burns, 1956) and ferns (Partanen, 1965) tend to support this idea.

Growth and Differentiation

Animals

Nuclear transplantation studies have helped to define the problems of genomes related to growth and development, especially in anuran embryogenesis. For example, diploid nuclei from late gastrula cells of *Rana pipiens* were transplanted to enucleated eggs by King and Briggs (1955); the larvae produced were found to be limited in their potential for differentiation. It was also found that for donor nuclei the later the stage of development, the more limited the differentiation. In other words, the events of ontogeny imposed a rather severe (and apparently permanent) restriction on the capacity for development.

The complementariness of maternal and paternal genomes was demonstrated in recent experiments by Subtelny (1965), who demonstrated that nuclei from androgenetic homozygous diploid blastulae of *Rana pipiens* developed into triploid froglets when transferred into nucleate ova; androgenetic diploid blastulae developed only as far as the abnormal post neural stage; hence, it appears that the haploid chromosome set of the ova contributed to normal development in the triploid heterozygous condition. This does not exclude the influence of maternal cytoplasm, however, since its integrity and composition depend in part on the resident maternal genome. A suggestion of this type of influence is seen in the work of Hadorn (Briggs and King, 1959) who showed that androgenetic haploid hybrids of *Triton palmatus* (enucleate egg) × *Triton cristatus* (sperm) normally die before showing specific characters; grafts of ectodermal por-

tions of gastrulae transplanted to *T. alpestris* survived, however, and showed skin protuberances typical of *T. palmatus*. This example of cytoplasmic maternal inheritance emphasizes the importance of female cytoplasm in influencing if not completely determining various characters.

Cell lines derived from human tissues show striking differences in growth, depending on whether they are diploid or heteroploid. The diploid cell lines undergo contact inhibition, show longer generation time, lack of growth in suspension culture, and lower cell densities, in

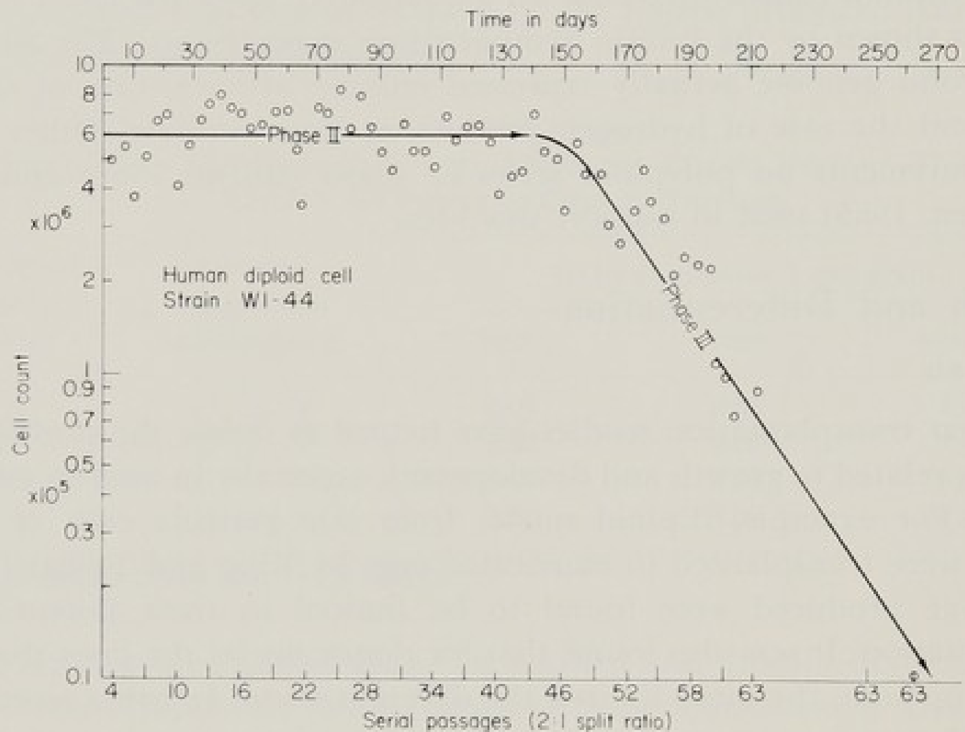

FIG. 3. The number of doublings of a diploid cell strain (WI-44) from human adult lung tissue from time of primary explant to phase III decline. After Hayflick (1965).

comparison with heteroploid cells which show the opposite characters (Eagle, 1965). In other words, certain restrictions on growth are still evident in the diploid cell lines. In heteroploid cell lines, precise control of all replication is lost, as well as control over special cell functions. The importance of these generalizations is emphasized by the work of Hayflick (1965). He showed that human diploid cell strains cease to divide not as a function of the number of subcultures, but according to the number of cell doublings (i.e., about 40–60 generations), as shown in Fig. 3. Moreover, it appears that the number of doublings occurring in adult lung tissue is lower than the number obtained from fetal lung tissue, which again emphasizes the fact that ontogenetic development does place increasingly greater restrictions on cellular capabilities, at least for growth.

Somatic cell genetics (Krooth, 1964) is rapidly becoming an important area of research, partly because of the implications for human genetics, for studies on differentiation, and for the recent advances in experimental somatic cell hybridization (Barski *et al.,* 1960; Ephrussi and Weiss, 1965). We can expect all the techniques of high resolution cell biochemistry to be utilized in the study of hybrid cells. Fusion of HeLa cells with rat lymphocytes, rabbit macrophages, and nucleated red blood cells of hens indicates a wide compatibility among cells of different species (Harris, 1965). The nuclei of these heterokaryons eventually fuse, and there is some evidence for stimulated RNA and DNA synthesis in differentiated cells following hybridization. These results are related to the earlier work of Moscona (1957) who obtained the reaggregation of dissociated embryonic cells of the chick and mouse. The application of these and other hybridization techniques to haploid cells of animals, and perhaps plants, can be expected in the future.

Plants

Totipotency is a character of diploid plant cells not generally shared by animal cells. Braun's (1959) demonstration of the recovery of normal plants from teratomatous crown gall tumors of tobacco was accomplished by isolating single cell clones of tissue, grafting into stems, and obtaining normal shoots from the implanted tissue. Induction of whole plants from diploid cells of carrots was achieved by Steward and co-workers (1964a,b) who suggested that free cells derived from young carrot embryos were virtually all totipotent if supplied with the appropriate nutrient. The free cells formed embryoids in a manner similar to that described for the zygote in the plant. Halperin and Wetherell (1964) have extended these studies to the wild carrot, presumably a more heterozygous plant than the inbred domestic carrot, and have found that callus tissues derived from various plant parts can also form adventive embryos. Regeneration from single cells of mosses (Ward, 1964) and ferns (Ito, 1960; Kato, 1964) as well as from tissue cultures of other plants such as *Ranunculus sceleratus* (Konar and Nataraja, 1965), *Chicorium endiva, Lactuca sativa,* and *Petroselinum hortense* (I. K. Vasil *et al.,* 1964) and from the female gametophytes of gymnosperms such as *Cycas revoluta* and *Zamia floridana* (La Rue 1948, 1954) and the derivation of tissues and plants from single isolated cells of tobacco by V. Vasil and Hildebrandt (1965) support the contention that plant cells are totipotent.

As far as differentiation is concerned, the gametophytes (n) of lower plants have been characterized by their lack of vascular tissue and this

has been considered typical of their haploid state. More recent work, however, has shown that fern prothallia of *Todaea barbara,* which usually possess no vascular tissue, will develop such tissue when grown on the proper concentrations of sucrose and indole-3-acetic acid (Wetmore *et al.,* 1964). Mosses lack vascular tissue, and there is no report of haploid sporophyte formation (Lal, 1963).

Apogamy, the formation of a sporophytic plant without gametic fusion, is a common occurrence in ferns and can be induced experimentally. One of the more revealing methods of induction is that utilized by Whittier and Steeves (1960) and Whittier (1964). Prothallia of *Pteridium acquilinum* were supplied with carbon in the form of sugar, and induced to form both haploid and diploid sporophytes; prothallia that obtained carbon from photosynthesis produced no apogamous structures. Bristow (1962) was further able to show that cultures of *Pteris cretica* could be maintained as gametophytic in red light and be induced to form haploid apogamous leaves in white light; the leaves were then induced to form callus or sporophytes according to the amount of sugar provided. Induced organization of a callus culture of *Lycopodium obscurum* also produced, presumably, haploid sporophytes (De Maggio, 1964).

It is obvious that some of our conventional ideas about the potential of haploid cells for growth and differentiation will have to be corrected. If a simple sugar can induce development of vascular tissue or complex formation of sporophytes, we are dealing with fundamental control mechanisms for form and function without the necessity of gametic fusion. This means that we know very little either about the mechanisms or about the limits of control of differentiation in haploids or diploids.

We have already noted that diploid animal cells appear to be limited in the number of duplications they undergo *in vitro* (Hayflick, 1965); plant cells do not appear to have this limitation. Relatively stable plant tissue cultures of diploid pea root callus were maintained by the use of a defined medium (Torrey, 1959); similar callus cultures on media containing 2,4-dichlorophenoxyacetic acid and yeast extract, changed from diploid to tetraploid within one week. Other work on tissue cultures of carrot (Mitra *et al.,* 1960) and *Haplopappus gracilis* (Mitra and Steward, 1961) grown on media supplemented with coconut water showed wide variations in chromosome number. The *Haplopappus* cultures showed some instances of somatic pairing and several haploid cells, and it was assumed that somatic reduction had occurred. Even single cell clones of tobacco tissue (Cooper *et al.,* 1964) were highly variable when grown on supplemented media. Spruce tumor tissue (Risser, 1964) grown on defined media

maintained a stable chromosome complement. Finally, the finding of Fox (1963) is important, since he observed that auxin- and cytokinin-independent strains of tobacco tissue possessed more stable chromosome numbers than strains requiring these compounds. A tentative generalization from these investigations seems to be that normal plant cells, like animal cells, can be grown indefinitely if grown as heteroploid populations on supplemented media; diploid lines of plant cells are maintained with this complement if grown on defined media, especially if the tissues are autotrophic for growth hormones.

Haploid Tissue Cultures of Higher Plants

Male Gametophyte (Pollen)

Tissue cultures derived from the pollens of seed bearing plants are of interest for several reasons: They facilitate studies on pollen physiology; they represent strains with genetically distinct characters when obtained from single pollen grains; they are useful for studies on differentiation and regeneration as extensions of the haploid male gametophyte.

Ideally, the best material for pollen tissue cultures would be genetically well-known plants such as corn, tomato, or *Oenothera*. Despite attempts by several investigators, however (see La Rue, 1954; Tulecke, 1959, 1963) the pollens of angiosperms have not yet been induced to proliferate as haploid tissue cultures. Excellent pollen tube development was obtained from pollen of more than 30 species of angiosperms grown on various media, but the only indication of unusual nuclear division was observed in the pollen tubes of *Antirrhinum majus* (snapdragon). Extra nuclei in addition to the expected tube nucleus and two sperm nuclei were observed (Fig. 4). This development occurred on a defined medium containing 0.1 mg/ml of kinetin and 2,4-dichlorophenoxyacetic acid, and no cell division was obtained. These results are in contrast to the vigorous development of pollen tubes of gymnosperms such as *Cupressus sempervirens* (Fig. 4). This pollen germinates and grows on defined media for weeks or months whereas angiosperm pollens usually grow only for hours or days. *Cupressus* forms clusters of sperm nuclei within the pollen tube and represents a more primitive, less specialized condition of the male gametophyte. Therefore, it is not surprising that gymnosperm pollens have been used successfully for the induction of androgenetic tissues.

The first tissue culture derived from pollen was obtained from *Ginkgo biloba* L. (Tulecke, 1953, 1957, 1960). Pollen grains distributed over the surface of nutrient media germinate and undergo either normal or ab-

normal development. Normal development proceeds from the four-celled microgametophyte (mature pollen grain) to the formation of two sperm cells, each about 100 μ in diameter and provided with cilia for motility. Mature spermatozoids have been obtained in cultures, but no motile forms have been observed. Abnormal development involves extra divisions of the tube nucleus or the multiplication of cells from the original

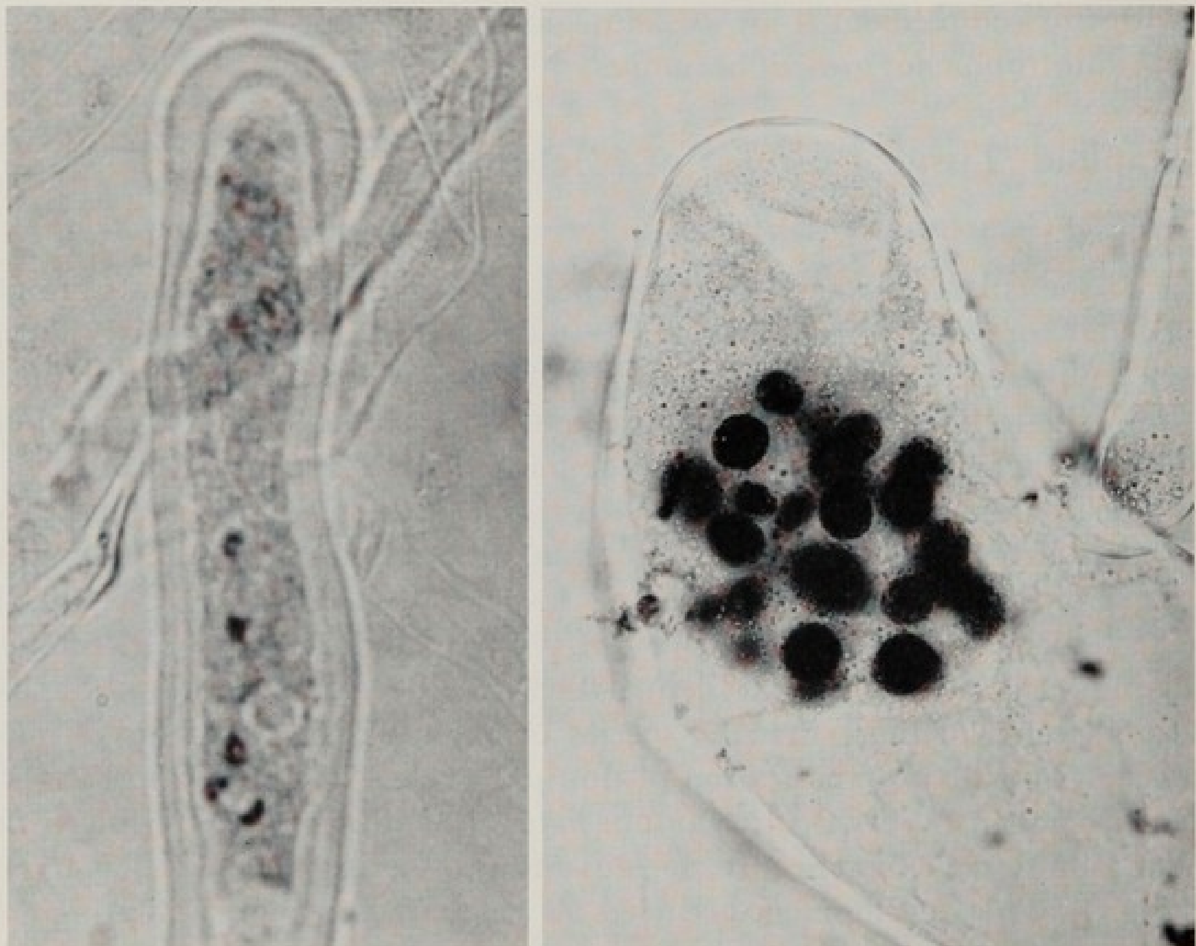

FIG. 4. Observations on pollen development *in vitro*. Left, pollen tube of *Antirrhinum majus* with three nuclei and dividing generative nucleus; right, cluster of male nuclei in pollen tube of *Cupressus sempervirens*.

generative cell. Eventually, after 2–6 months growth, visible tissues may be seen in the pollen cultures. Some phases of this development are shown in Fig. 5. Other haploid tissue cultures of *Taxus* sp. (Tulecke, 1959), *Torreya nucifera* Sieb. and Zucc. (Tulecke and Sehgal, 1963), and *Ephedra foliata* Boiss. (Konar, 1963) have been derived from gymnosperm pollen. As shown in Fig. 6, the tissues have distinctive growth patterns. Cells from the pollens of *Ginkgo* and *Ephedra* divide mitotically to form daughter cells in a manner typical of plants. The cells of the *Taxus* tissue

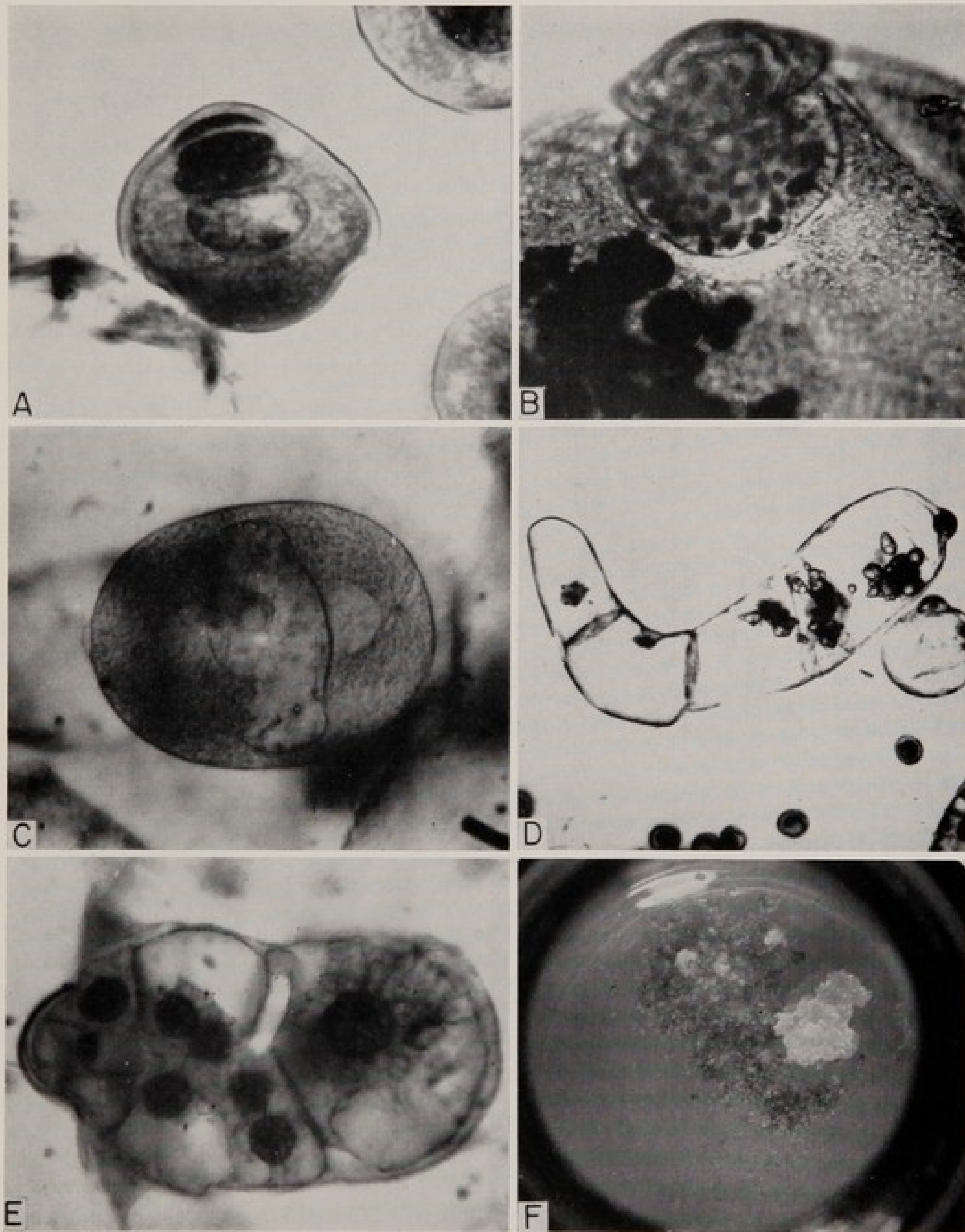

FIG. 5. Development of *Ginkgo biloba* pollen *in vitro* (normal): A, mature pollen grain; B, starch grains in plastids of generative cell; C, sperm mother cell prior to division to form two motile sperm; (abnormal): D, septate pollen tube; E, cluster of cells derived from the generative cell; F, tissue forming from pollen. The original pollen grain is about 25 μ in diameter and the sperm mother cell approximately 150 μ.

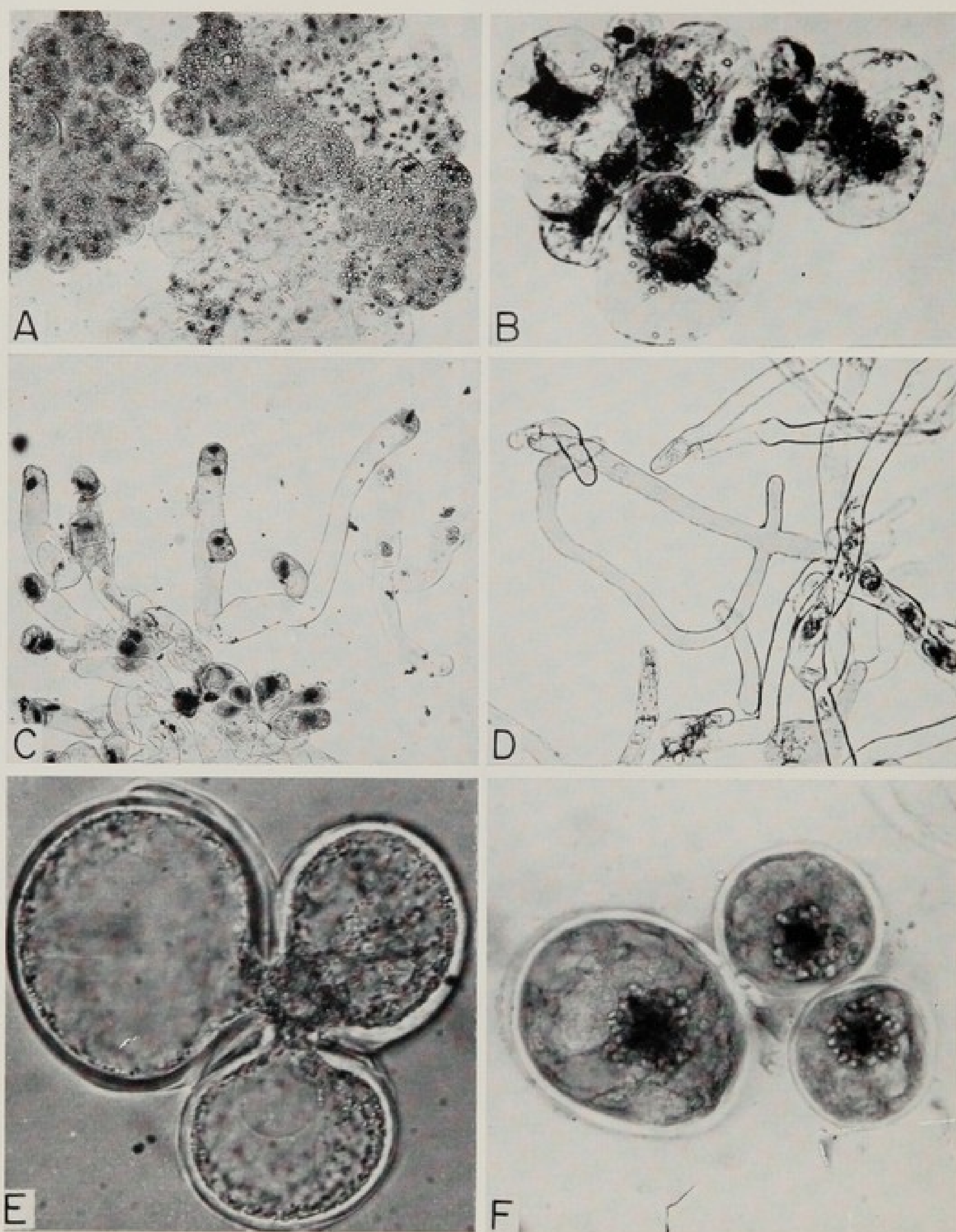

FIG. 6. Characteristics of haploid tissues derived from pollen: A, squash preparation of *Ginkgo* tissue; B, cells of tissue from *Ephedra* pollen from Konar (1963); C-D, *Taxus* tissue showing pollen-tube type cells with nuclei often located terminally; E-F, yeast-like budding of *Torreya* tissue with derived uninucleate cells shown on the right. The average cell size is about 50–200 μ.

grow in an elongated manner typical of growing pollen tubes; the nucleus and cytoplasm are localized at one end, and when division occurs unequal cells are formed, some of which are branched. The most unusual form of growth is observed in the cells of the tissue from *Torreya* pollen. Although they are very slow in growing, the cells divide by yeastlike budding. All of these tissues are white in appearance and show amyloplast development; chlorophyll synthesis is apparently blocked. No differentiation beyond meristematic and parenchymatous cells is observed.

Observations on the chromosome complements of the tissues derived from the pollen of *Taxus, Ginkgo,* and *Torreya* indicate that haploid cells are still present in the tissue after several years in culture; polyploid cells, including diploids and tetraploids, are also found in all tissues. The polyploid cells and nuclei are larger, but show no capacity for specialized differentiation.

Female Gametophyte

The female gametophyte of angiosperms is a much reduced structure, the embryo sac; in contrast, the same tissue in gymnosperms is large enough to bear two or more archegonia and to serve as a food reservoir for the developing embryo. Because of the generous size of the gametophytic tissue in *Ginkgo biloba* and because a haploid male gametophyte tissue was already available, a tissue culture of female origin was sought and obtained (Tulecke, 1964). The male and female gametophyte tissues are therefore available for comparative studies on maternal inheritance, plastid function, and differentiation.

The tissue culture of the female gametophyte proliferates well on a defined medium, contains chloroplasts, and is made up of several types of cells; chromosome counts indicate that the tissue is basically haploid. Some cells resemble those in the intact plant in their ability to form chloroplasts, store a resinous material, deposit a crystalline substance, differentiate tracheids, or continue growth as meristematic cells (Fig. 7). Absorption spectra of extracts from the female gametophyte and the tissue culture indicated that the pigments formed *in vitro* are similar to those found in the plant (Fig. 8). Quite often, female gametophyte tissues or cotyledons of gymnosperms are green while still within the seed. Apparently, all the precursors of pigment synthesis are formed and transported to the tissues and chlorophyll is formed in the dark before the seeds germinate. The female gametophyte tissue culture is normally green, but loses its chlorophyll when placed in the dark; growth proceeds equally well in the light and in the dark. On exposure to light the dark-grown

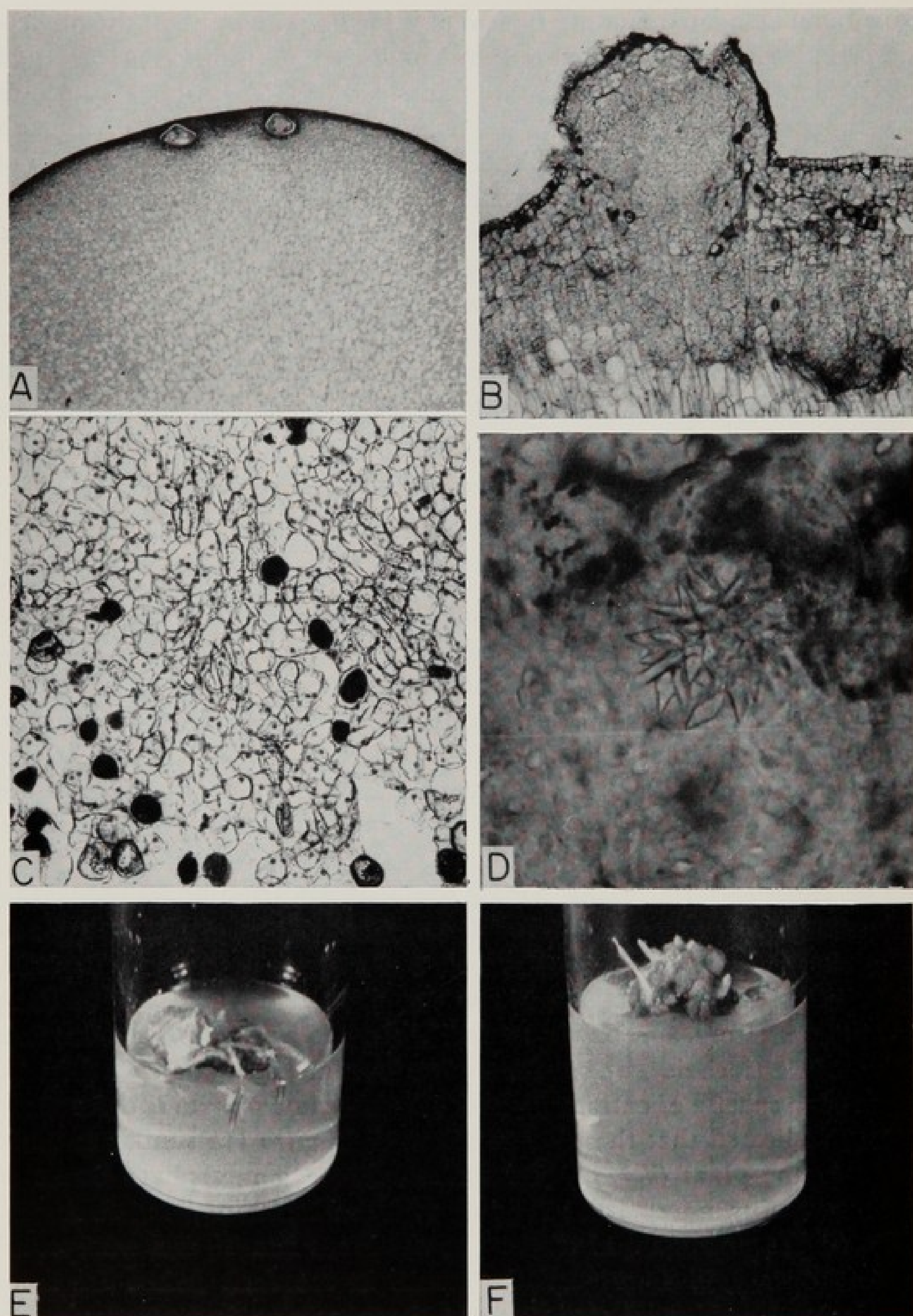

FIG. 7. Characteristics of a tissue culture derived from the haploid female gametophyte of *Ginkgo biloba* L.: A, section through the apical end of the female gametophyte

tissue slowly regains its color. It is not yet known what precursors of chlorophyll are needed for greening in the dark, but the tissue should be useful for studying this phenomenon.

The differentiation of roots and shoots has been observed in some experiments with the female gametophyte tissue; examples are shown in Fig. 7. A total of 15 roots was observed originating from callus masses cultured on several different media. Abortive shoots were seen in only one

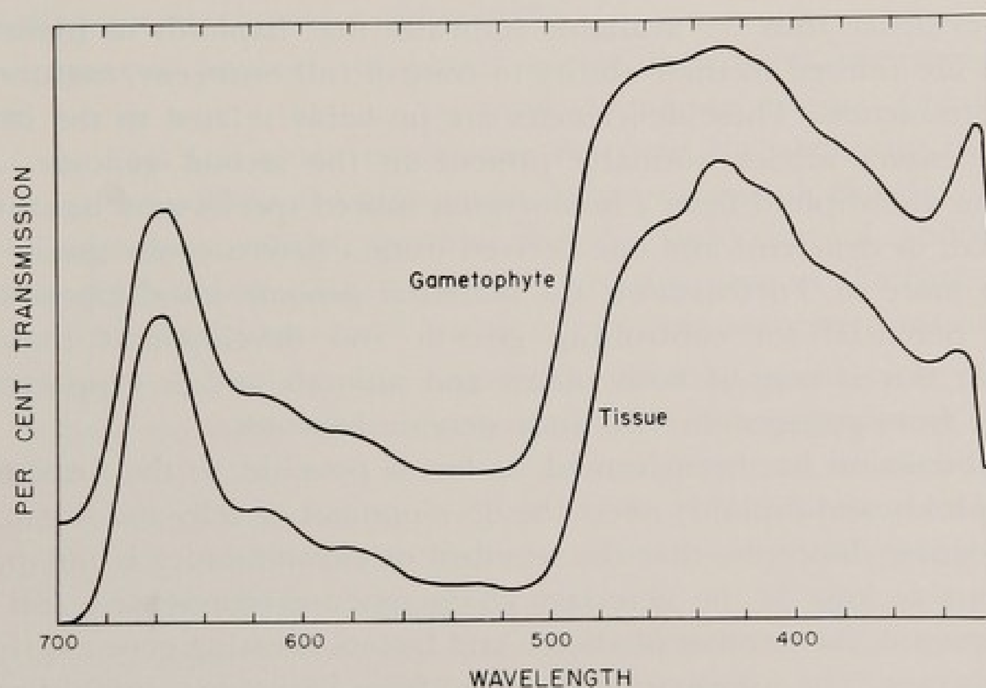

FIG. 8. Absorption spectra of acetone extracts of *Ginkgo biloba*. Upper curve, the female gametophyte from the tree; lower curve, the tissue derived from the gametophyte.

culture in which five such organs were formed. Indirect evidence from cell and nuclear size indicated that the structures were haploid, but conclusive chromosome counts still need to be obtained. Microscopic examination of the roots and shoots revealed vascular strands; the ageotropic shoots possessed scalelike leaves and an abortive apical meristem. Several basal media and various supplements were used, but the most important component appeared to be purines such as the cytokinin, kinetin. Further

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showing immature archegonia in the primary explant; explants lacking archegonia develop tissues equally well (the gametophyte is about 1 cm in diameter); B, growth of tissue from the primary explant; C, parenchyma, resin, and tracheidlike cells of the established tissue culture; D, crystal inclusion of cell from gametophyte tissue culture similar to inclusions in mature leaves; E, root formation; F, abortive shoot development.

work is needed to establish the quantitative and qualitative requirements for root and shoot formation.

In contrast to the specialized cell types found in the female gametophyte, no differentiation has ever been observed in the male gametophyte tissue of *Ginkgo* grown on a wide variety of media.

Conclusion

The evidence thus far available indicates that haploids of higher organisms are limited in their ability to control full ontogeny, maturation, and reproduction. These deficiencies are probably related to the lack of complementary alleles normally present in the second genome. As a result, an autodiploid from a homozygous inbred species may be stunted, abnormal, or deficient; and one derived from a heterozygous species may be even more so. Furthermore, the maternal genome usually possesses a greater potential for controlling growth and development than the paternal; this is true of both plants and animals and is supported by evidence from gynogenetic and androgenetic haploids.

Our discussion has been limited, as far as possible, to the question of how haploidy and diploidy affect the development of cells and organisms. We recognize, however, that the number of chromosomes is not highly important as long as the necessary characters are represented, but that gene sequence, the number of alleles, and factors affecting gene expression are important. The information obtained from haploids is useful because the state of complementary alleles has been largely eliminated. We have attempted to reduce the complexity of cells and organisms to a simpler, readable state; in this sense, haploids are useful and are likely to be increasingly helpful as more become available for experimental use.

Homozygosity is clearly the advantage offered by the haploids as experimental material. As potential breeding stock (i.e., as autodiploids), they are valuable gene pools of a certain uniformity. Hybrids of autodiploids of related species may show some pairing of chromosomes and these affinities are indicative of homologous chromosomes or segments. Hu (1960), for example, performed a karyotype analysis on two haploid species of rice and found similar bivalents and trivalents; he concluded that the five known discontinuous characters which separated the two species were not distinguishable at the chromosome level; hence, the species were relatively closely related. Studies of this type can be helpful in taxonomic investigations and in problems of phylogeny.

The usefulness of haploids is restricted by their relatively infrequent occurrence. Their use in breeding forest trees (Nei, 1963) or corn (Chase,

1963), for example, will depend on improved techniques for inducing haploid plants. Two possible approaches for obtaining haploids would be the regeneration of plants from haploid tissue cultures and the induction of somatic reduction followed by regeneration. These or other methods for obtaining haploid offspring would save time in achieving homozygosity and would aid genetic studies and breeding programs. The advantages, however, need to be weighed against the hazards of the generally poor development of haploids.

Androgenetic haploids of plants are of infrequent occurrence even among species where they are reported (*Nicotiana, Crepis, Antirrhinum,* and *Zea*). This fact alone emphasizes the need for obtaining tissue cultures from the pollens of angiosperms. When successful, this development should provide excellent material for the induction of androgenetic haploids. Such tissues could be used in investigations of the basic mechanisms which control male and female expressions and functions in plants.

From an evolutionary viewpoint, the diploid-haploid cycle of plants and animals has been selected as a mechanism for engendering variability. In the same way, many flowering plants appear to be evolving from diploid to polyploid lines; the original diploids may become extinct and eventually new diploids may arise from the existing polyploids (Raven and Thompson, 1964). These pseudohaploids actually provide a means for genetic variation, selection, and subsequent adaptation in recurrent cycles, in a way not greatly different from the normal haplontic-diplontic life cycles described in mathematical terms by Lindenmayer (1964). The polyploids may be better balanced genetically, more heterozygous, and isolated from diploids; their rate of evolution, however, is slower and their mutants are concealed.

Little reference has been made here to cytoplasmic inheritance, since this subject has been discussed by other participants in this symposium (see chapters by Schiff and by Srb). However, the evidence that plastids and mitochondria contain DNA (Gibor and Granick, 1964) suggests that haploid plants and tissues will be especially useful in studying cytoplasmic inheritance. In particular, haploid tissues should be useful in investigating the functions of organelles in cells with a single genome. Some of the questions of maternal and paternal inheritance may be answered by this approach.

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Cell and Tissue Interactions in the Reproduction of Cell Type

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Introduction

The phrase "reproduction of cell type" may, to some students of development, seem to contain an internal contradiction. We classify cells on the basis of the specialized cytoarchitecture, biochemistry, and physiology of the state we define, somewhat arbitrarily, as fully differentiated. Yet cells which have reached this stage rarely divide. To be sure, under the special circumstances which initiate regeneration, many types of "fully" differentiated cells do participate in the initial proliferative stages. In so doing, however, they rapidly lose at least those cytological features by which they can be recognized as definitive types of cells.

Therefore, in discussing the manner in which populations of a particular type of cell are augmented, we are in essence treating two distinctly different processes. We must first consider the replication of some progenitor cell and second, the processes by which these progenitor cells become specialized.

During vertebrate embryogenesis, and indeed in the immature animal as well, both processes—cell division and cytodifferentiation—occur concomitantly within the same tissue or organ. It has been assumed that these two processes do not occur in the same cell, but not until relatively recently has it been possible to examine this assumption critically.

Using radioautographic techniques, several investigators have been able to identify the proliferative members of such developing populations (Stockdale and Holtzer, 1961; Wessells, 1964a; Makela and Nossal, 1962; Young, 1962; Leblond, 1964; Kitiyakara and Angevine, 1963). The consensus has been that DNA synthesis occurs only in the progenitor cells: those cells in which the specialized products specific to the cell type cannot be detected (but see Leblond, 1964).

To date, the most extensive investigations into the proliferative phases associated with the production of differentiated cell types are those of Leblond and his colleagues (recently reviewed in Leblond, 1964). This group has been engaged in examining the growth of a variety of organs in the postnatal rat using a combination of biochemical, radioautographic, and cytological techniques. On the basis of such studies, Leblond (1964) classifies cell populations within the organism as static, expanding, or renewing. Static populations are homogeneous groups of cells in which no mitotic activity can be detected and in which the DNA content remains constant. Although neurons fall into this category, other neural elements (e.g., spongioblasts) proliferate, but the frequency of mitosis declines with time. Expanding populations are those in which scattered mitoses can be detected in quantities which account for the increase in total DNA content. In such populations (kidney, pancreas, and adrenal) DNA accumulation is quite rapid immediately after birth but gradually approaches constancy. In these organs, mitosis is not restricted solely to connective tissue cells. Paired labeled nuclei are found in adjacent differentiated parenchymal cells (e.g., pancreatic acinar cells, proximal convoluted tubule cells of the kidney, and follicular cells of the thyroid). In the last category, the renewing population, an intensive cell proliferation occurs, providing cells in numbers far exceeding those required to account for any increase in the DNA content. In such populations this high rate of cell production is balanced by cell loss. Cell loss may occur either by emigration (e.g., hematopoietic tissue, reticuloendothelial system, and seminiferous tubules) or by cell attrition (e.g., digestive tract, pleural epithelium, and epidermis).

Those tissues in which provision is made for continuous replacement are generally those which suffer the greatest attrition in the organism. Continuous renewal of these tissues is an adaptation which extends longevity by preventing the accumulation of defects. In the renewing populations mitotic activity is restricted to a stem cell line frequently set apart anatomically from the specialized cells of the tissue. In the epidermis, for example, the basal layer of cells consists of actively proliferating cells which are cytologically and biochemically unspecialized. Individual cells of this basal layer lose their attachment to the underlying basement membrane, presumably because they are forced up into the more superficial layers of the epidermis. Only after losing their association with the basement membrane do these cells initiate keratin synthesis.

Thus, during embryogenesis and continuing through postnatal life into adulthood, the reproduction of cell type occurs through an intermediary—the progenitor cell. We know that these progenitor cells do

not contain detectable quantities of the specific products characteristic of their ultimate fate. We do not know, however, the extent to which their repertoire of developmental responses may have been restricted. From a variety of experimental situations, it is clear that the basal cell of the epidermis and of specialized epidermal structures such as sebaceous glands and hair can undergo specializations characteristic of other epidermal regions (Montagna, 1956). The well-known transformation of keratinizing epidermis into mucoid-secreting epithelium by vitamin A is only one example (reviewed by Fell, 1961).

Whether the basal cells of an adult epidermis can be channeled into specializations other than those in the epidermal repertoire is unknown.

Since these particular transformations are reversible in the tissue as a whole, a distinction is often drawn between the responses of basal cells and true "differentiation." Some authors (cf. Mercer, 1961) prefer to consider them "modulations" (see Weiss, 1950), that is, different physiological states of a single type of cell. The confusion perhaps results from the fact that no distinction is made between the basal cell population and the individual differentiated descendants of this population. In fact, a basal cell is always channeled into one epidermal specialization or another; when the environment is changed, it is not the specialized cell that reverts. On the contrary, it is another cell, still in the basal layers, still unspecialized, which is influenced by the new conditions (Fell and Mellanby, 1953). In no sense does the same cell modulate between two different specialized states (see Weiss and James, 1955, p. 392).

Regulation of Stem Cell Differentiation

Stem cell populations such as those represented by the basal layers of stratified squamous epithelium are in a steady state. The production of new cells balances the continuous loss of cells from the upper layers of the epithelium. By removing one superficial layer at a time, Pinkus (1951, 1952) was able to demonstrate that the removal of as few as four layers of the stratum corneum of epidermis invoked a significant increase in mitotic activity. To maintain a steady state, loss of cells from the basal layer must occur at a rate comparable to the rate at which new cells are produced. After a cell moves out of the basal layer it no longer divides, but presumably as a response to its changed location, initiates the synthesis of proteins characteristic of cornified epithelial cells.

An older view held that the shift to synthetic activities of specialization was due to a differential division of a stem cell. This view has been examined by Leblond and his associates (1964) and found to be untenable.

According to the hypothesis, the division of a basal cell would be in some manner unequal. One daughter cell would remain in the basal layer and continue to serve as a stem cell while the other daughter cell would emigrate to progressively more superficial layers. In the process this cell would become progressively more keratinized, die, and be sloughed off. Such a mechanism, however, is not compatible with the distribution pattern of labeled nuclei in radioautographs of esophageal epithelium at progressively longer intervals after the administration of tritiated thymidine. During the first 12 hours, all the labeled cells complete mitosis, yet an insignificant number of labeled cells is found in the spinous layer. After 12 hours, the number of labeled cells in the spinous layer gradually rises. By using criteria such as the proximity of labeled cells and relative grain density of cells, Leblond and his collaborators were able to recognize the daughter cells of a single mitotic division. The migration of one or *both* daughter cells of a pair occurs at random. There is thus no evidence that one cell invariably remains behind and that the other differentiates.

Differentiation does not appear to be related to some special feature of the preceding mitosis. Leblond suggests that migration is initiated simply by the random displacement of a basal cell resulting from population pressures. We would suspect that the decisive cue which triggers differentiation is a function of new environmental relationships. It is not known whether loss of contact with the basement membrane or the influences emanating from surrounding spinous cells is the critical factor.

In the differentiation of an epithelial stem cell there is a suggestion that differentiation is directed by the cellular environment, as in embryogenesis. Although less dramatic than the classic examples of embryonic induction, interactions of a similar nature may continue to play a role in late embryonic, postnatal, and adult life (see Auerbach, 1964). Such interactions include any influence exerted locally by a dissimilar cell which alters and stabilizes biosynthetic activities and eventually the cytological appearance of the responding cell. Although tissue interactions between cells of dissimilar type are more usual, it is not unreasonable to assume that a more differentiated cell might influence the development of a less differentiated cell of the same series. For example, a spinous cell might influence a displaced basal cell.

The Cellular Environment and Stem Cell Differentiation

The persistence of an inductive interaction into late embryonic and perhaps early postembryonic life is best exemplified by recent investiga-

tions into the growth and development of the lens. The lens contains both a population of differentiating cells and a segregated germinal stem cell line. In this respect, the lens is analogous to the epidermis with which it shares a common embryonic origin. Although differentiation leads ultimately to death of the cell in both tissues, differentiated cells in the lens form the definitive lens and are retained. The cuboidal germinal cells form an epithelium over the anterior surface of the lens. As individual germinal cells pass the margin of the lens, they start to elongate and to synthesize a (third) specific lens protein, gamma crystalline (Papaconstantinou, 1964, 1965; Takata *et al.*, 1965). Two lines of evidence indicate that as these cells pass the margin they are subjected to influences emanating from the posterior tissue of the eye.

If the lens of a chick embryo is removed and rotated through 180 degrees before it is replaced in the eye, it will still regulate in time to form a more or less normal lens even if the operation is performed at a relatively late stage (J. L. Coulombre and Coulombre, 1963). The germinal epithelial cells, most of which now face the back of the eye, stop dividing and start to elongate; only those epithelial cells at the lens margin continue to proliferate. These cells now migrate both anteriorly and posteriorly from this proliferative marginal zone. The former re-establish a new lens epithelium while the latter elongate and contribute in normal fashion to the growing lens. The interpretation of these results is that the germinal cells, exposed by the operation to the posterior chamber of the eye, respond to influences they would not normally encounter until they had passed the equator of the lens.

An even more direct demonstration of the influence of posterior tissues of the eye upon the developing lens has been obtained using culture techniques. Epithelium from the lens of 13-day-old mouse embryos fails to differentiate further when isolated in culture. When the epithelium is combined with isolated neural retina, however, either directly or with an ultrathin Millipore filter interposed, a complete new lens with oriented fibers is formed (Auerbach, 1964; Muthukkaryppan, 1964). Philpott and Coulombre (1965) found that isolated lens epithelium in culture will palisade to a limited extent, but only if the medium contained a protein supplement (serum or ascitic fluid). Such cultured epithelium failed to differentiate further when grafted into the coelomic cavity of 5-day-old chick embryos. These epithelial cells, when transplanted back into a lentectomized eye, continued to elongate and differentiate into lens fibers of more normal appearance.

The neural retina, derived from that part of the optic vesicle which

induces the primary lens placode, apparently continues to influence lens development in later stages. Earlier investigators had suggested that lens induction is a protracted event (reviewed by A. J. Coulombre, 1965a,b). Stone (1954) has in fact shown that the neural retina influences the formation of a lens from the dorsal iris in Wolffian regeneration in the adult urodele. The extent to which these influences exerted at later stages are analogous to the primary inductive event is problematical but not untestable.

Many of our concepts of embryonic induction have changed with the passage of time and with the availability of new analytical approaches. We know now, for example, that cellular contact is not an indispensable requisite of all inductive events (Grobstein, 1953). It would seem that another time-honored criterion of embryonic induction, namely, that the effect persists in the absence of the inducer, needs re-evaluation. This may be true of the original cells which are induced, but in the further growth of the induced structure differentiation of additional primordial cells may be dependent upon a persistent source of directive influences. Such influences may be provided by derivatives of the original inductive tissue as seems to be the case in the lens-neural retina dependency discussed above. It is also possible that such influences are exerted by the induced cells themselves. It may be recalled that Mangold and Spemann (1927) demonstrated that medullary plate could, in turn, induce medullary plate in gastrula ectoderm. Moreover, specific areas of the medullary plate impose regional specificity on the responding gastrula ectoderm.

Experimental Approaches to Embryonic Induction

The problem of embryonic induction or tissue interaction has been most profitably examined over the past 12 years by an approach which greatly simplifies the experimental design of earlier studies. Moreover, it permits manipulations which were previously either impossible or extremely difficult. These techniques, introduced and developed by Grobstein (1953, 1954), have been applied to a group of inductive events which occur between epithelial primordia of a variety of organs and their subjacent mesenchymal components. Briefly, the two components are separated with trypsin and fixed in apposition on either side of an ultrathin (20 μ) filter with pores of microscopic dimensions. Culture in this fashion permits the normal interaction to occur under conditions which lend themselves to a variety of analytical approaches.

These studies have fundamentally altered our concept of embryonic

induction. By far the most important contribution has been a demonstration that inductive effects can be transmitted across short distances. This means that in certain instances there is a substance that can be isolated, purified, and identified. If this can be accomplished, the mechanism of action can be studied.

Grobstein's studies demonstrate that some macromolecular material is entrapped in the filter (Grobstein, 1954, 1963). This material is sensitive to proteolytic enzymes and exhibits some of the staining properties characteristic of the general class of mucopolysaccharides. Radioautographic studies indicate that this macromolecular material crosses the membrane (Kallman and Grobstein, 1965); one of these macromolecules has been identified (Kallman and Grobstein, 1964, 1965). None of the materials has as yet been identified with the active factor or factors of the mesenchymal cell. One encouraging finding recently reported is that a high concentration of embryo juice or of a particulate fraction of this extract replaces the effects exerted by mesenchymal cells when present in the medium (Rutter *et al.,* 1964). The embryo contains a high proportion of mesenchymal cells and it is entirely reasonable that the extract should be active. This finding is encouraging because it indicates that the factor (or factors) is relatively stable. If it were highly unstable, the prospects of its eventual purification would be reduced. In yet another inductive system—the spinal cord-chondrogenic interaction—an active extract has been prepared from pooled embryonic spinal cords which can replace the living effector tissue (Hommes *et al.,* 1962; Strudel, 1962; Lash *et al.,* 1962; Lash, 1963). The identity of the active component is in doubt presumably because of the difficulty of purifying the trace quantities which are available.

Cytodifferentiation in Cell Culture

Our own inclination has been to study differentiation in still simpler systems. All the single events which occur during the development of an organism are simple and comprehensible. The complexity that exists, exists only in the mind of the investigator. The value of a simple system is that we can more readily ask a clear and simple question.

In developing a test system, we are fully aware that natural limits exist. The number of components could be reduced to a point at which the system no longer retains those properties we are interested in studying. Cytodifferentiation, as Grobstein (1962a) has cautioned, occurs *in vivo* "in the context of a higher level of integration and interaction between

diverse tissues." At what time in its history and to what extent can a cell be isolated from the integrative influences which might regulate its differentiation in the organism? It was to this question that we initially addressed our experimental approach.

We did not anticipate that the initial question would lead ultimately, as it has, to a consideration of cell and tissue interactions. Our major concern was at first with the more crucial issue of whether a single isolated cell could survive, divide, and still retain its capacity for differentiation in culture. The cumulative experiences of many investigators suggested that this was not possible. The many theoretical arguments based on this experience, however, were often constructed without regard for the empirical nature of culture approaches. The issue involving stability of differentiative function in culture has been discussed at length elsewhere (Konigsberg, 1963); additional positive evidence for stability has been discussed more recently (Eagle, 1965). At the present time, it seems of little value to ruminate over issues which are rapidly losing their controversial content.

The choice of tissue, skeletal muscle, was in retrospect not an ideal one because unlike the epitheliomesenchymally derived organs used in the transfilter approach we had no clear insight into the roles of the interacting cell types. It did have the advantage, however, that the major cell type of the tissue, the muscle "fiber" was easily characterized on the cytological as well as molecular level.

The first phases of the investigation dealt with the behavior in culture of relatively large numbers of single cells liberated by trypsinization from embryonic chick leg muscle (Konigsberg *et al.,* 1960; Konigsberg, 1960, 1961a). Although the tissue was taken from advanced embryos, it still contained large numbers of mononucleated cells which were, by this criterion, relatively undifferentiated (Fig. 1); these mononucleated cells are the predominant type in cell suspensions prepared from embryonic muscle tissue. Whether such disorganized cells, dispersed on a glass surface in a liquid culture medium, continue to differentiate and form typical skeletal muscle fibers was the initial question; we found that they did. Hence, tissue organization per se is not indispensable to the normal differentiation of skeletal muscle cells.

Studying the characteristics of these cultures, we can recognize two distinct phases. During the first period, the cells attach and spread on the surface of the petri dish, and then multiply rapidly, forming a confluent sheet of cells by the third or fourth day (Konigsberg, 1961a). By gross inspection at least, the cells are devoid of any indication of frank

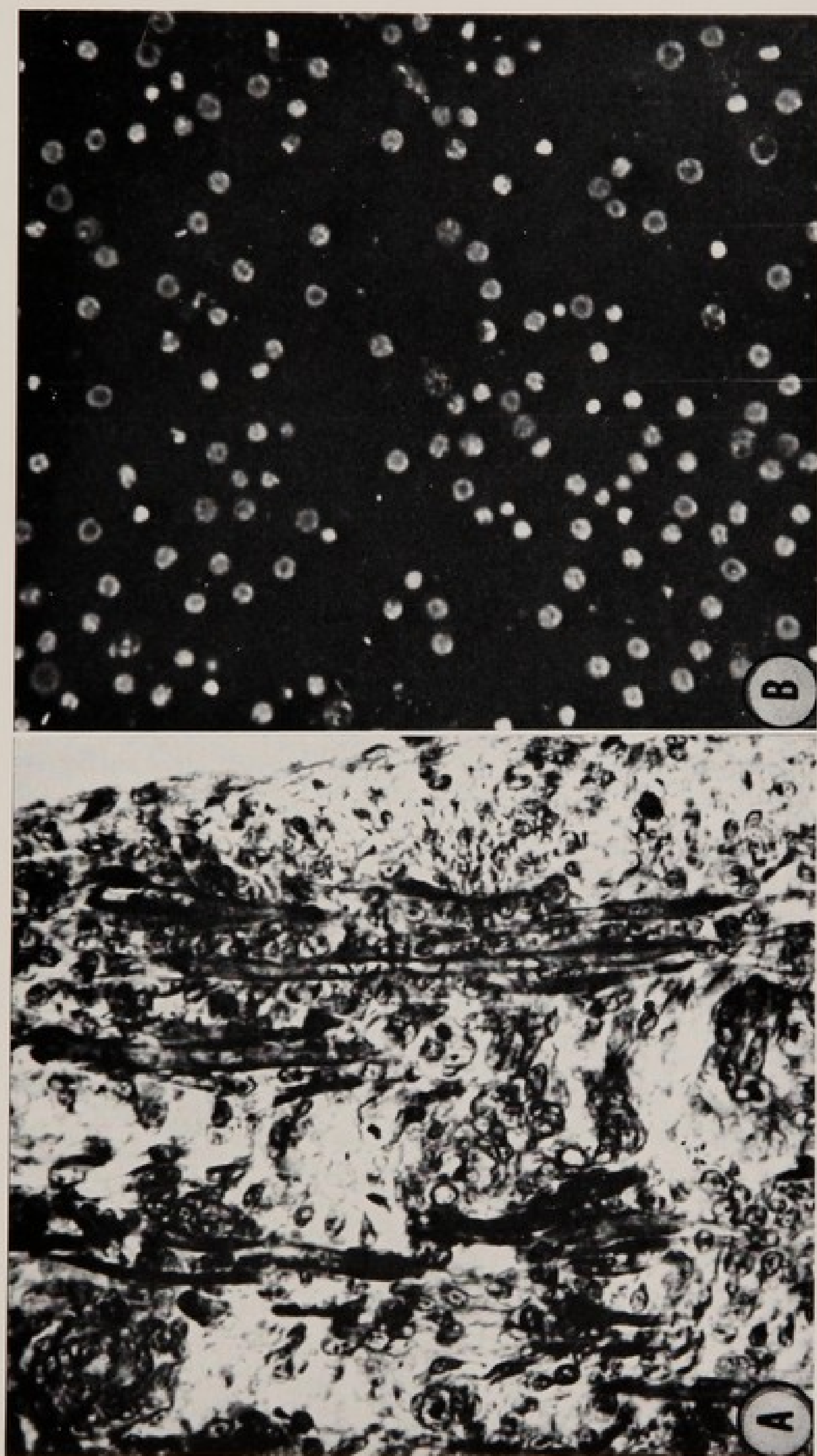


Fig. 1. A. Sagittal section through a thigh muscle from a 12-day-old chick embryo. Fixed in Allen's B-17; stained in iron hematoxylin. Elongated multinucleate myotubes are clearly in evidence. In addition, large numbers of mononucleated cells, presumably both myoblasts and fibroblasts, are also present. B. Cell suspension prepared by trypsinization (10 minutes, at 37°C in 0.05% 1-300 trypsin) of a mince of leg muscle from a 12-day-old chick embryo. Photographed in phase contrast.

muscle differentiation during most of this period. As confluency is approached, however, long, multinucleated, ribbon-like cells resembling myotubes rapidly appear (see Konisberg, 1965b). These are the primitive muscle fibers observed *in vivo*. Subsequently, the multinucleated cells can be positively identified by virtue of their spontaneous contractility and the presence of cross-striated myofibrils. Concomitant with the attainment of confluency, a pronounced transition from exponential growth to a very slow linear increase in cell number (DNA per culture) was observed (Konigsberg, 1961a).

Population Density and Cytodifferentiation

These three phenomena—confluency, cessation of growth, and the appearance of multinucleated myotubes—seemed to be closely correlated. Moreover, the time of appearance of all three could be shifted simultaneously by varying the size of the inoculum.

This correlation did not prove, of course, that causal relationship existed between the attainment of confluency and the formation of multinucleated cells. If such a relationship did exist, however, there seemed to be two sound and testable alternative mechanisms by which it might operate. The attainment of confluency might promote myotube formation simply by reducing intercellular distance, thus increasing the frequency of random cell-to-cell collision. This seemed a perfectly plausible explanation since the best available evidence at the time suggested that multinuclearity occurred by a process of successive cell fusions (reviewed in Konigsberg, 1965b). Alternately, as confluency was approached, the increased cell density might produce appreciable alteration of the medium as a result of the metabolic activities of the cultured cells. The possibility of such a mechanism had in fact been demonstrated by the work of Earle and his associates (Sanford *et al.*, 1948) who used "conditioned" medium, and by Puck and his collaborators (Puck and Marcus, 1955) who employed "feeder" layers.

To discriminate between these alternatives "parabiotic" cultures were set up in which the same medium circulated over both a sparsely seeded and a densely seeded culture. Myotubes formed in both cultures at approximately the same time, and earlier than the event occurred in sparsely seeded controls. The next question was obvious. Were alterations in the medium of a stable nature or was the continued presence of a large number of cells required? Not only was the question obvious, but the experimental design was clear.

Replicate cultures of the same cell suspension were subsequently fed either with freshly prepared medium or conditioned medium recovered from older cultures which had already attained confluency. Under the conditions of these experiments, myotube formation occurred 24 hours earlier in conditioned medium (Konigsberg, 1962).

Conditioned Medium and the Development of Muscle Clones

We had learned that the normal tissue architecture was not indispensable to the subsequent differentiation of myoblasts *in vitro*. We also knew that differentiation was promoted by some influence of a high population density and that this influence was transmitted via the medium. Consideration of whether this population density-dependent influence was at all related to the course of normal myogenesis *in vivo* was for the moment postponed. Whether physiological or not, was it sufficient to permit the growth and differentiation of single isolated myoblasts?

An elegant series of techniques for cloning animal cells in culture had been devised by Puck's group (Puck *et al.*, 1956). With relatively minor variations, these were applied to freshly isolated embryonic muscle cells. After a rather tedious empirical examination of the parameters of the procedure for preparing conditioned medium, we devised a schedule which yielded consistent results (Konigsberg, 1963). It was abundantly clear that properly conditioned medium was indeed adequate to support the growth and differentiation of colonies of cells descended from single isolated myoblasts.

Small numbers of dissociated cells from embryonic muscle were inoculated into petri plates containing conditioned medium and cultured for approximately 2 weeks. At the end of this period, a gross examination of fixed and stained cultures revealed the presence of two distinctly different colonial forms (Fig. 2). By microscopic examination, those colonies which have a fibrous appearance can be identified as muscle colonies using the same criteria by which skeletal muscle cells are recognized *in vivo* (Fig. 3). That these colonies can indeed originate from a single cell was investigated both by following the entire process and by physically confining single cells within small glass cylinders (Konigsberg, 1963). Such isolated colonies contain large numbers of the same multinuclear, cross-striated muscle fibers observed earlier in mass cultures and in embryonic muscle fixed and sectioned at the appropriate stage. In addition, we also observed mononucleated cells within the same colony. These mononucleated cells,

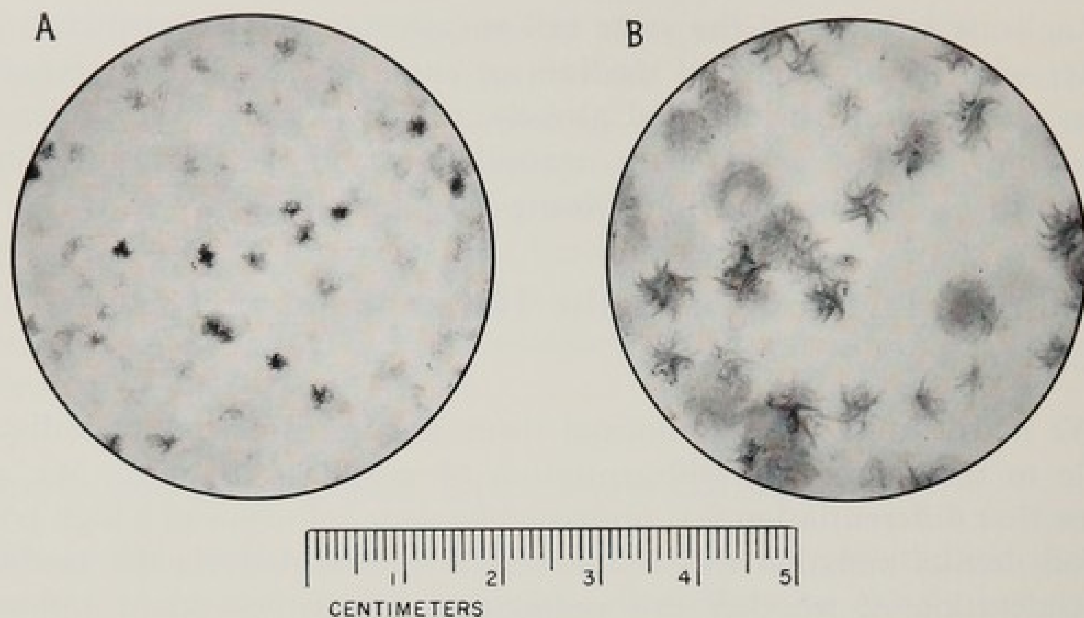


FIG. 2. Two petri plate cultures seeded with 200 cells each. Fixed in Bouin's and stained in Meyers' hematoxylin. A. Fixed after 6 days of culture. By gross inspection alone muscle colonies cannot be distinguished from fibroblast colonies at this time. B. Fixed after 13 days of culture. Two distinctly different types of colonies can be observed. The fibrous colonies with irregular edges can be identified as colonies containing differentiated skeletal muscle cells (Fig. 3). The regular discoid colonies are composed of fibroblastic cells.

if selected (by the use of antimycin A which specifically destroys the differentiated muscle fibers) and transferred by trypsinization to petri plates containing conditioned medium, can recapitulate the entire process of growth and differentiation and form a macroscopic muscle colony (Konigsberg, 1965a).

The Conditioning Process: Possible Mechanisms

Satisfied that conditioned medium was adequate to support the growth and differentiation of isolated myoblasts, we turned to a consideration of the kind of influence densely populated "farm" cultures could be exerting on the conditioned medium. The two most likely alterations of the medium are either detoxification or the relief of specific nutritional deficiencies. Fisher and Puck (1956) have demonstrated that feeder layers can perform both functions. Recently, Eagle and his collaborators (Eagle and Piez, 1962; Eagle, 1963) have emphasized the relationship between cell density and the nutritional requirements of cultured cells. They found that since cells are usually cultured under conditions in which the extracellular space (the culture medium) is disproportionately larger than it is

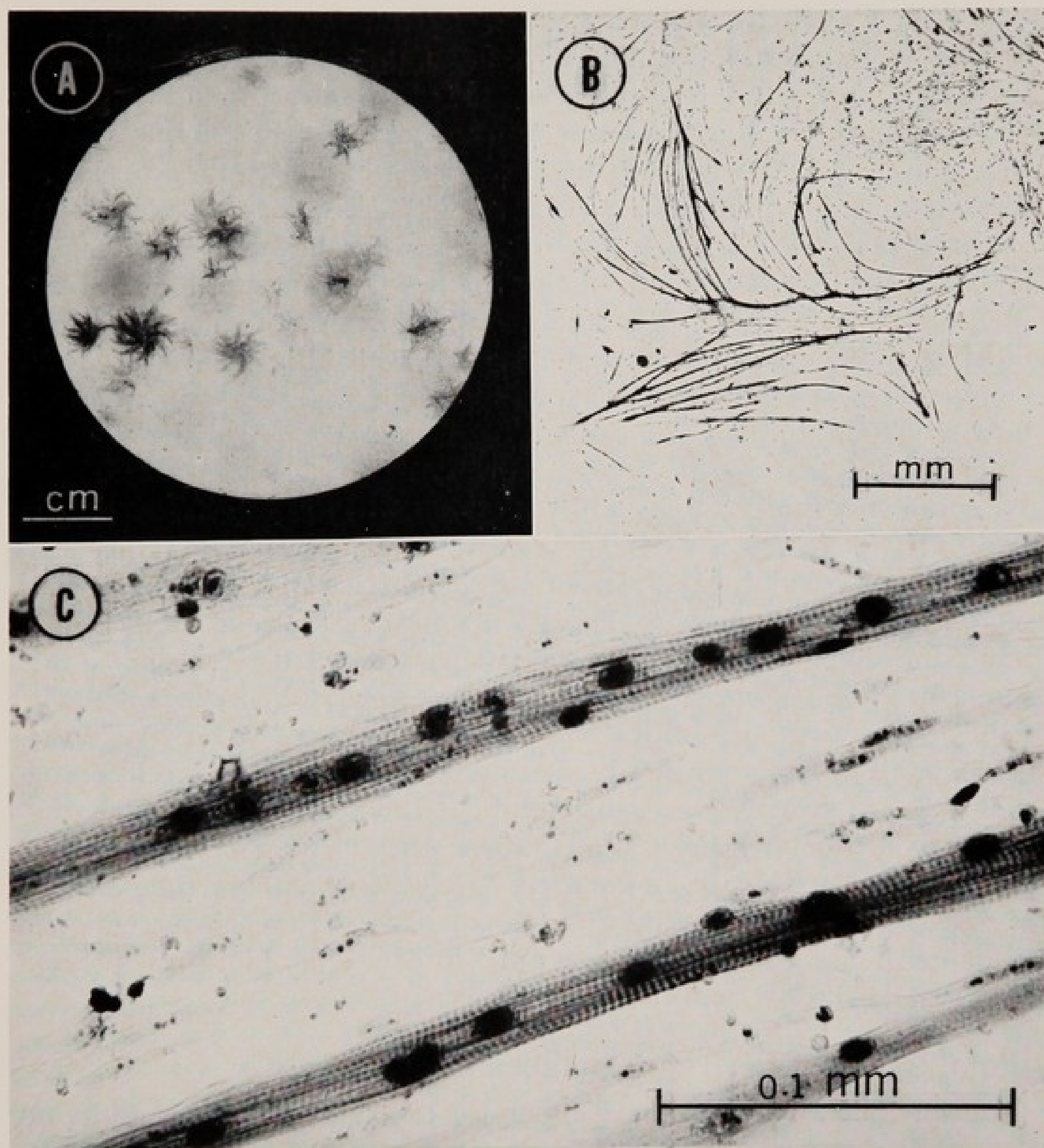


FIG. 3. At progressively higher magnification the distinctive fibrous-appearing colonies can be identified as colonies of skeletal muscle cells by the same criteria used to identify skeletal muscle *in vivo*. A. Petri plate culture fixed after 16 days in Zenkers' and stained in iron hematoxylin. B. At higher magnification, the network of myotubes of a small muscle colony can be distinguished. The colony figured here is approximately 5 mm from the center, at 10 o'clock, in the petri plate in A. C. An area of the myotube network of B at still higher magnification. These cells, like skeletal muscle cells *in vivo*, are multinucleate. The cross-striated pattern, typical of voluntary muscle, can also be observed in these cells.

in vivo, losses of cell metabolites to the extracellular space severely limit survival and growth. Examining several amino acid requirements of cultured cells, they found that even in cases in which the cell could synthesize a specific amino acid, it frequently required an exogenous supplement of that amino acid. At sufficiently high cell densities, however, such supplementation becomes unnecessary. At these high levels, the particular amino acid accumulates so rapidly that it reaches concentrations sufficient to maintain the necessary intracellular concentration. In addition to fairly ubiquitous metabolites such as amino acids, we could not ignore the possibility of similar phenomena regulating the availability of cell products more specific to particular cell types. Although Eagle's group employed pure cell strains in their studies, our farm cultures contained mixed populations of at least two types, presumptive fibroblasts as well as myoblasts. We could not dismiss the possibility of cross feeding among different types of cells.

There was certainly no dearth of possible mechanisms or of molecular species which could conceivably play a role in the effects exerted by conditioned medium. The only clue we had was that if it were simply the supplementation of one or more factors, these would have to be reasonably stable. To be sure, one possible approach was a methodical investigation of the levels of all known metabolites both before and after conditioning the media. Eventually, we might even hope to be able to reproduce conditioned medium synthetically. However, the life-span of man is still roughly "three score years and ten" (Ps XC:10). Furthermore, the prospect of engaging in such research was not a palatable one even if success were guaranteed. Instead, we have attempted to define the biological parameters of the effects of conditioned medium.

Biological Parameters of Conditioning

During the past year, we have attempted to reduce the formidability of the problem by asking well-defined questions of more limited scope. One question with which we have been concerned was whether conditioned medium represented an absolute requirement of the isolated myoblast. We had known for some time that even in unconditioned medium a small variable number of aberrant muscle colonies developed (Konigsberg, 1961b, 1963). These, of course, might have been formed by a small number of "variant" myoblasts which were not dependent upon conditioned medium (Figs. 4 and 5). It was equally possible, however, that during the cloning procedure the colonies themselves were to some degree conditioning the medium in which they were growing.

We reasoned that if cross feeding did occur between colonies, crowding the colonies closer together should enhance the exchange. Series of cultures were set up in which the number of inoculated cells varied between 100 and 1200 cells. Parallel series were run in conditioned as well as unconditioned medium. Plotting the number of muscle colonies against the total number of colonies after a 2-week growth period (Fig. 6) indicated that crowding did not increase the yield of muscle colonies cultured in conditioned medium. By progressively crowding the colonies closer together in unconditioned medium, however, progressively higher per-

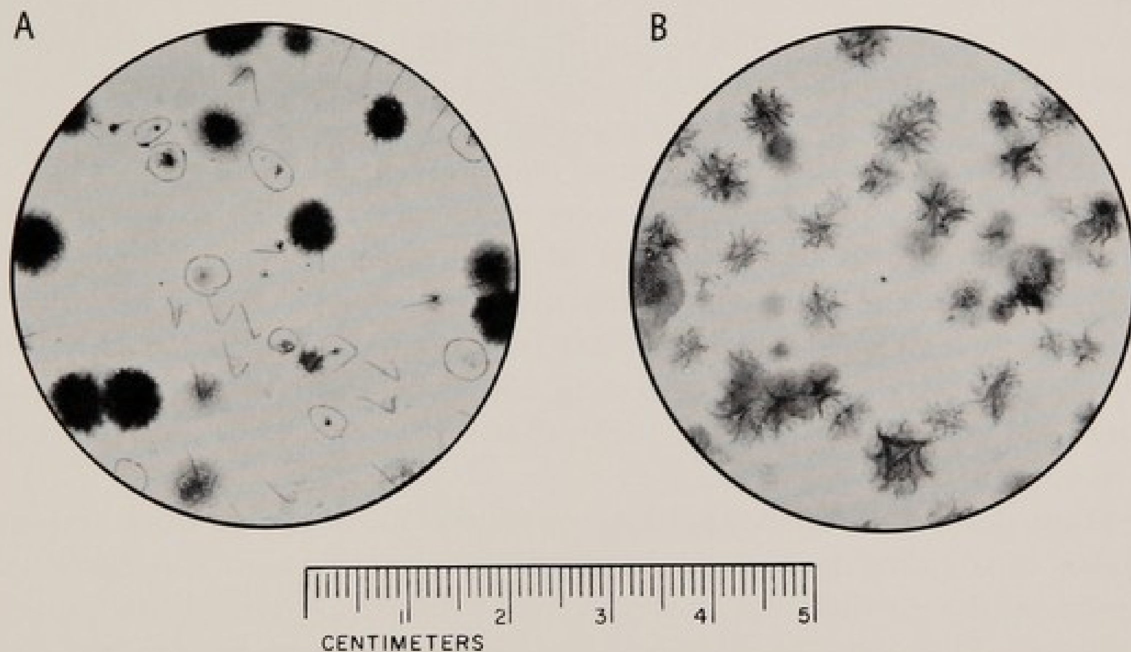


Fig. 4. A comparison of colony types which develop in unconditioned medium (A) and in conditioned medium (B). Both plates were fixed after 13 days of culture. The circles and checks on the petri plate in A are an aid in scoring colony types. The circles mark the locations of colonies containing identifiable myotubes. Compare the minute size of these colonies to the muscle colonies on plate B.

centages of muscle colonies were obtained. We concluded that some interaction between adjacent colonies occurs in unconditioned medium leading to the development of muscle colonies which either would not have formed at all or would have been scored as fibroblastic. Since the same effect is not observed in conditioned medium, it may mean that with conditioning already optimal, the maximum number of muscle colony progenitor cells have expressed themselves in this medium.

These observations at least established the plausibility of the thesis that the few aberrant muscle colonies which develop in unconditioned medium are also dependent upon conditioning. Another line of investiga-

tion suggests that the development of muscle clones is entirely dependent upon the use of conditioned medium.

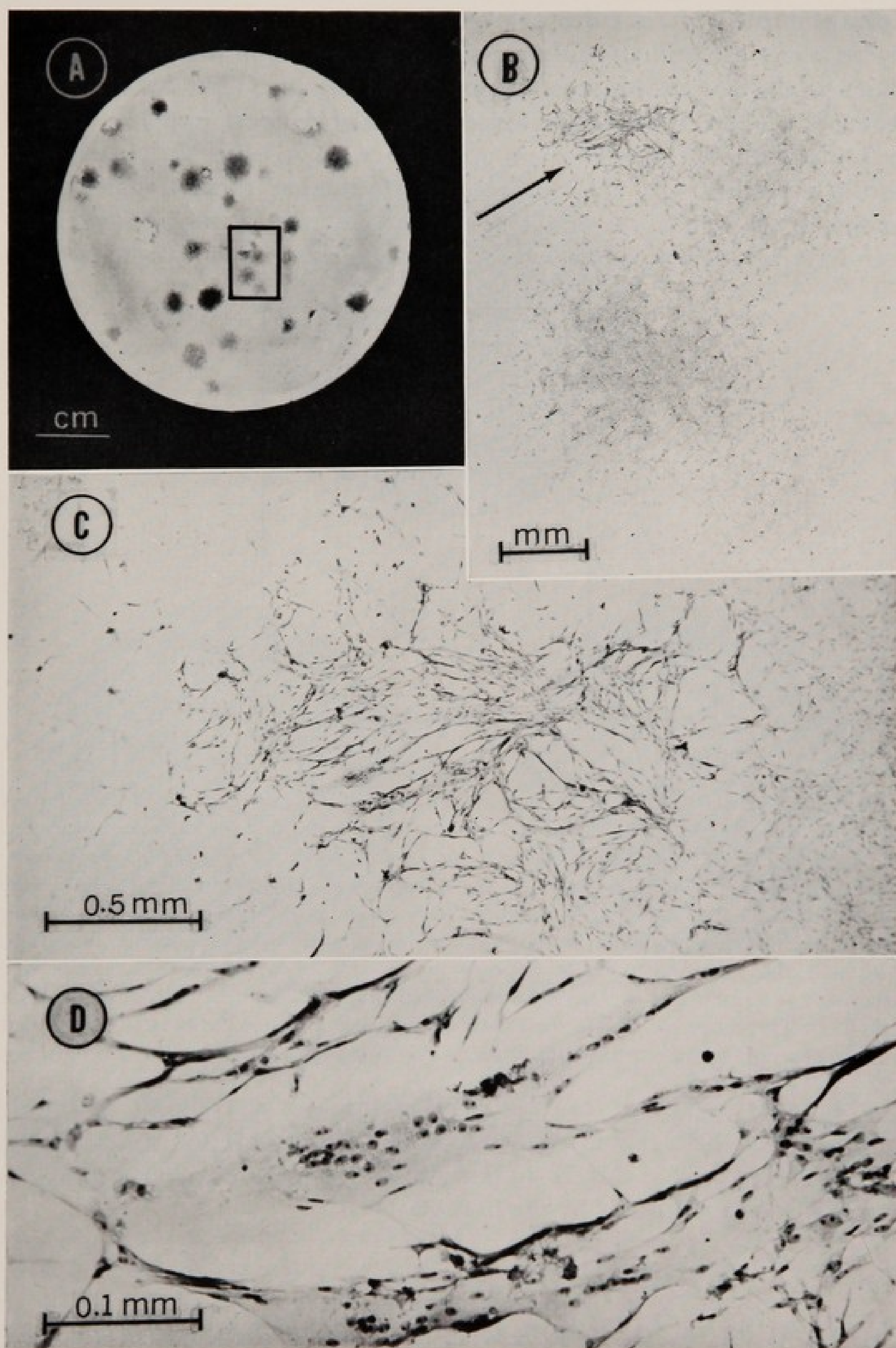
In a series of experiments designed to test another question, we prepared cell suspensions by trypsinization of single colonies of either muscle or fibroblastic type. These suspensions were transferred to individual petri plates containing conditioned medium and incubated for the standard 2-week period. Such second generation colonies breed true to the parental type (Figs. 7 and 8).

We now took cell suspensions prepared from single muscle colonies and pipetted half the cells into a plate containing conditioned medium and half into unconditioned medium. The transfer cultures in unconditioned medium were usually completely devoid of colonies or, on occasion, contained a few minute colonies which could not be identified as muscle. In contrast, the plates with conditioned medium contained large numbers of muscle colonies which frequently were extremely crowded. Although both plates had received heavy inocula of muscle cells, only in conditioned medium are these cells competent to form colonies. Some fibroblastic colonies at least will clone in either type of medium (Fig. 9). That all fibroblastic colonies do not behave in a uniform fashion may indicate, as suggested earlier (Konigsberg, 1963), that these colonies do not represent a homogeneous population.

From these experiments we may conclude that some colonies of fibroblastic cells either do not require conditioned medium or can effectively condition the medium themselves. None of the cells obtained from muscle colonies will form clones unless conditioned medium is provided. Apparently they cannot condition the medium sufficiently to establish themselves. This suggests that conditioning is primarily a function of the fibroblasts.

We knew, in fact, that conditioned medium could be prepared by using farm cultures consisting almost exclusively of fibroblastic cells. Medium so prepared was as effective as the medium conditioned by mixed populations of muscle cells and fibroblasts.

FIG. 5. Microscopic examination of one of the minute muscle colonies which develop in unconditioned medium. A. Culture fixed and stained after 10 days. The outlined area is magnified in B. B. Arrow indicates the small muscle colony visible within the area outlined in A. Note that this colony is in close proximity to a colony of fibroblastic cells. This is frequently although not invariably true of muscle colonies in unconditioned medium. C. and D. At higher magnifications the multinucleate myotubes can be seen. The pattern of cross striation can be observed in unstained preparations of such cells examined in polarized light (Konigsberg, 1961b).


A
cm
B
mm
C
0.5 mm
D
0.1 mm

Such fibroblast farm cultures are seeded with cell suspensions prepared by trypsinizing primary cultures which have grown to confluency. In these confluent cultures most, if not all, of the myoblasts have fused to form multinucleated fibers. These differentiated fibers fragment during trypsinization, but most of them are removed by passing the suspensions through bolting silk. It is problematical whether these fragments are viable. In any event, secondary cultures prepared in this manner contain relatively few myotubes. According to our knowledge of the optimal

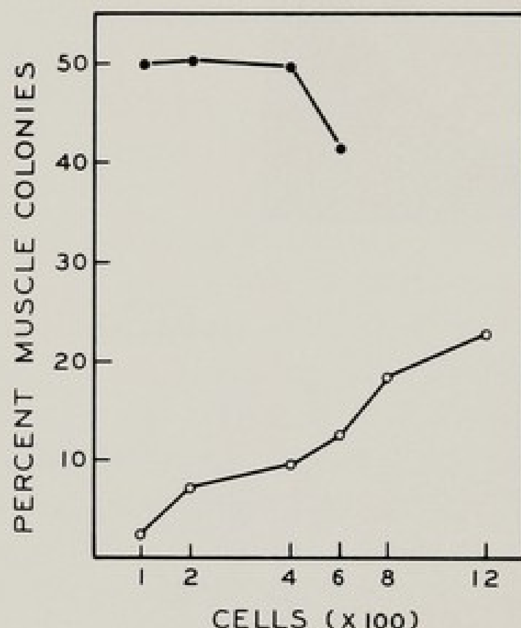


FIG. 6. Variation of the percentage of muscle colonies (ordinate) as a function of inoculum size (abcissa). Cultures were inoculated with freshly suspended cells within the range indicated. They were cultured for 13 days with a change of medium every 3 days (no fourth feeding was given). The medium was either conditioned (●——●) or unconditioned (○——○). Throughout this range of inoculum sizes the plating efficiency either remained unchanged or declined slightly with increasing inoculum. Although the percentage muscle colonies in unconditioned medium increased with increasing inoculum this was not true of the percentage muscle in conditioned medium (see text). The range of inocula used with conditioned medium could not be extended beyond 600 cells because of the difficulties in counting such crowded cultures accurately.

cell numbers required to condition medium effectively, it is highly unlikely that these few myotubes could be responsible for conditioning.

These observations added support to the premise that conditioning is accomplished principally by the fibroblasts.

The Critical Period

We next turned to the question of whether there was a specific time during the development of a muscle clone during which conditioned

medium was required. Replicate cultures were set up in conditioned medium with small numbers of freshly isolated muscle cells. At 3-day intervals the medium was replaced, and at each interval a group of cultures which had been fed up to that time with conditioned medium was now

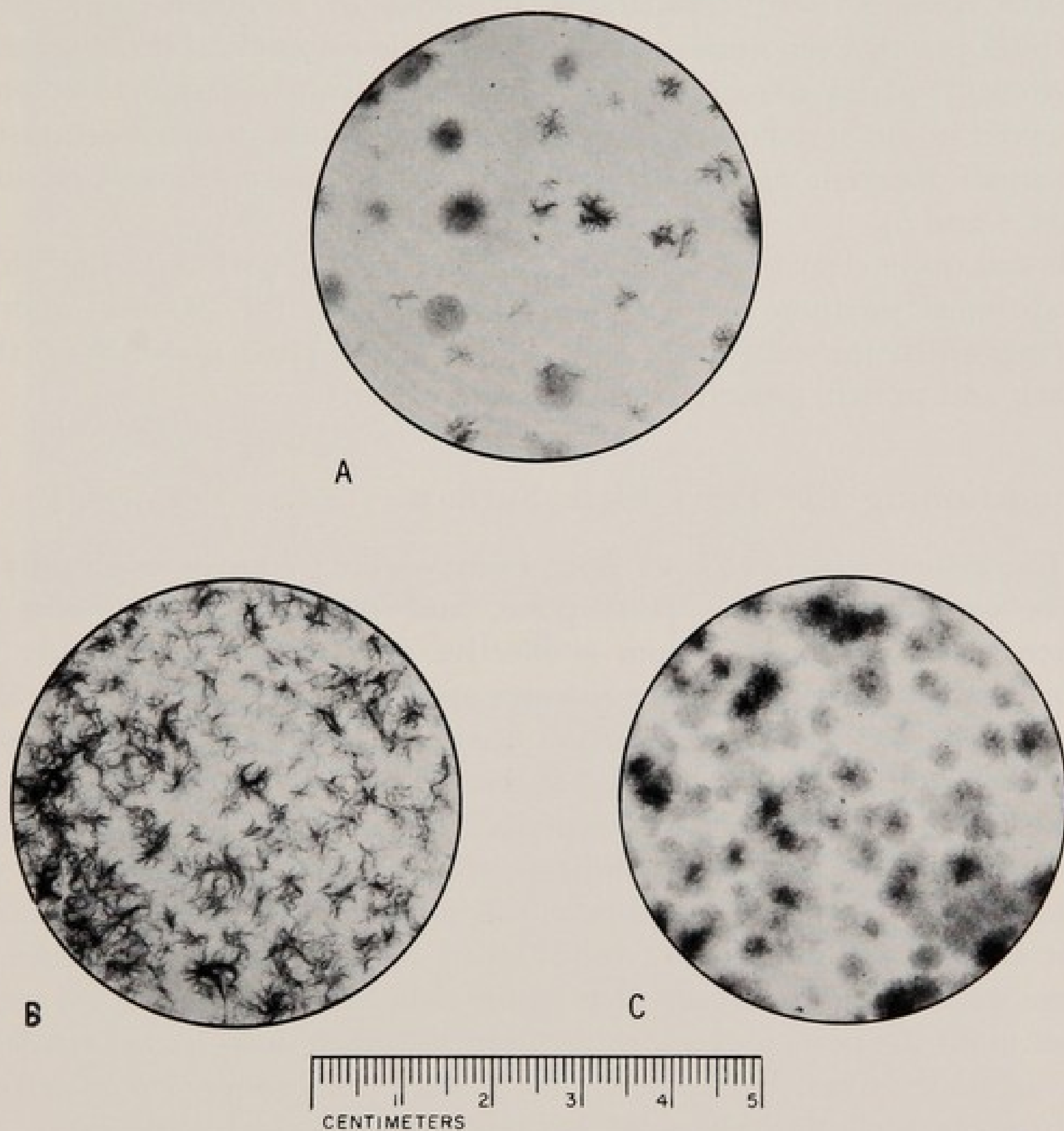


FIG. 7. Second generation subclones of muscle and fibroblastic colonies in conditioned medium. A. Sister culture of the primary plates used as the source of the colonies transferred. This culture was fixed on the same day (day 12) that the transfers were made. The petri plate in B was inoculated with cells suspended by trypsinization of a single muscle colony. C. Subclones of a single colony of fibroblastic cells.

switched to unconditioned medium. These "switched" plates were then carried in unconditioned medium for the remainder of the standard 2-week incubation period. An examination of the plates clearly indicated that colonies of muscle cells cultured in conditioned medium for 3 days were indistinguishable from those cultivated for the entire 2-week period in the

same medium. Moreover, with some batches of conditioned medium an interval as short as the first 24 hours in culture was sufficient.

In the cultures initiated in conditioned medium and transferred to unconditioned medium, both the plating efficiency and the percentage of muscle colonies were comparable to those of full-term conditioned medium plates. Even more striking, the muscle colonies on the short-term "switched" plates were identical to the large sworling muscle colonies observed in our standard conditioned medium plates, in sharp contrast to the small aberrant muscle colonies observed in unconditioned medium (Figs. 4 and 5).

It was quite clear that there was indeed a specific period during which conditioned medium was required. This period, the 24-hour interval during which the cells settle to the floor of the petri plate where they attach and stretch, proved to be surprisingly short.

Conditioning the Petri Plate Surface

The system with which we have been working consists essentially of three components: cells, petri plate, and conditioned medium. Apparently, conditioned medium is effective even if present for only the short period during which the cultures are initiated. It seemed logical to inquire whether the first interaction occurs between cells and medium or between medium and the surface of the petri plate. We wanted to determine whether the brevity of the period during which the cells required conditioned medium might indicate that the petri plate surface was altered in some way by exposure to this medium and whether, once this new surface was established, conditioned medium was dispensable. (It seems pertinent to point out that in our laboratory it is standard procedure to fill the petri plates with medium and store them in the incubator for an hour or two before use. This practice was adopted originally to reduce the time the cells need be exposed to normal atmosphere and room temperature.)

To test this premise it would be necessary to treat petri plates with conditioned medium, in the absence of cells, and then substitute un-

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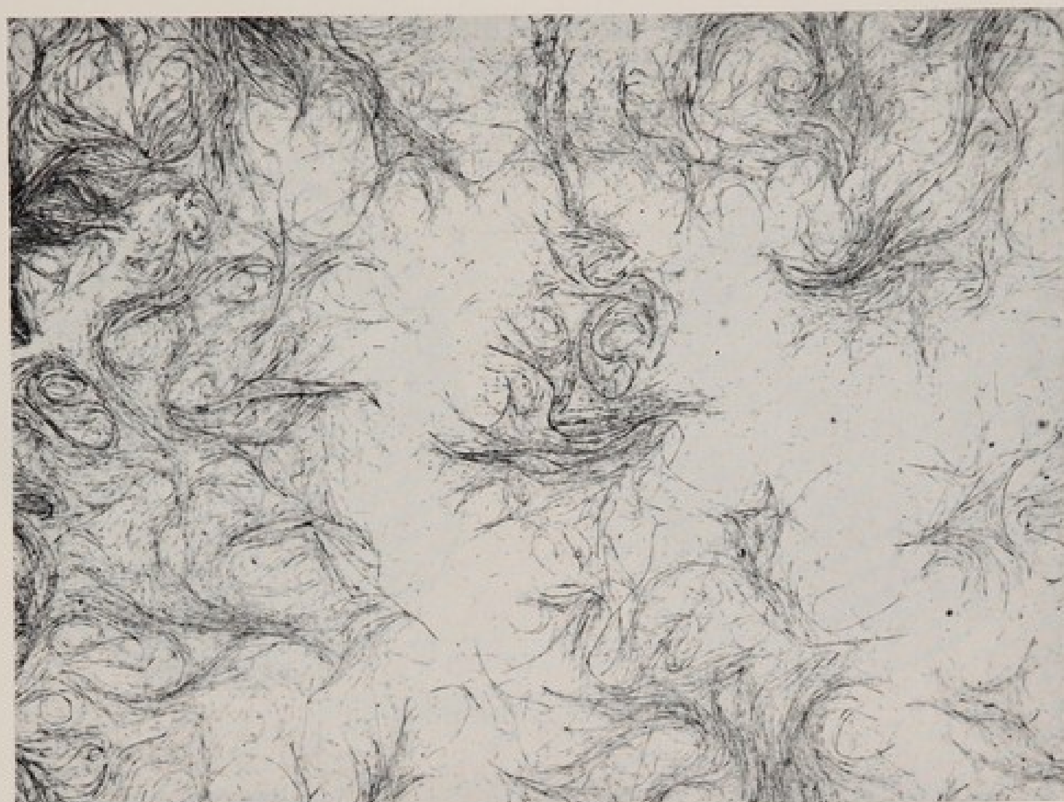
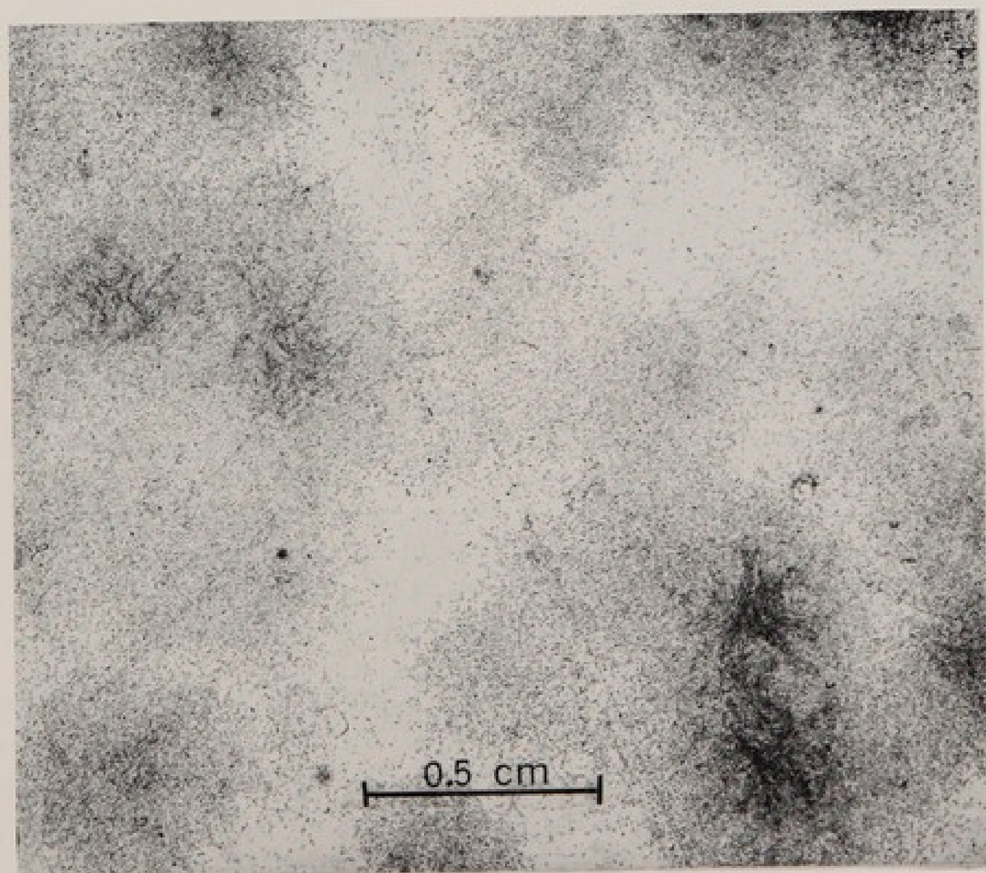
Fig. 8. Higher magnification photomicrographs of areas of the second generation subclones in Fig. 7. B. Second generation colonies subcloned from a single muscle colony. C. Daughter colonies of a primary colony of fibroblastic cells. Sworls seen in the center of some of these colonies (upper right) are composed of mononucleated cells. They are due, most probably, to multilayering in colony centers.



B



C

conditioned medium before inoculating the cells. Therefore we preincubated plates with conditioned medium for a period of 3 days at 36.5°C in an atmosphere of 5% CO_2, aspirated off the conditioned medium and replaced it with 2 ml of unconditioned medium. These

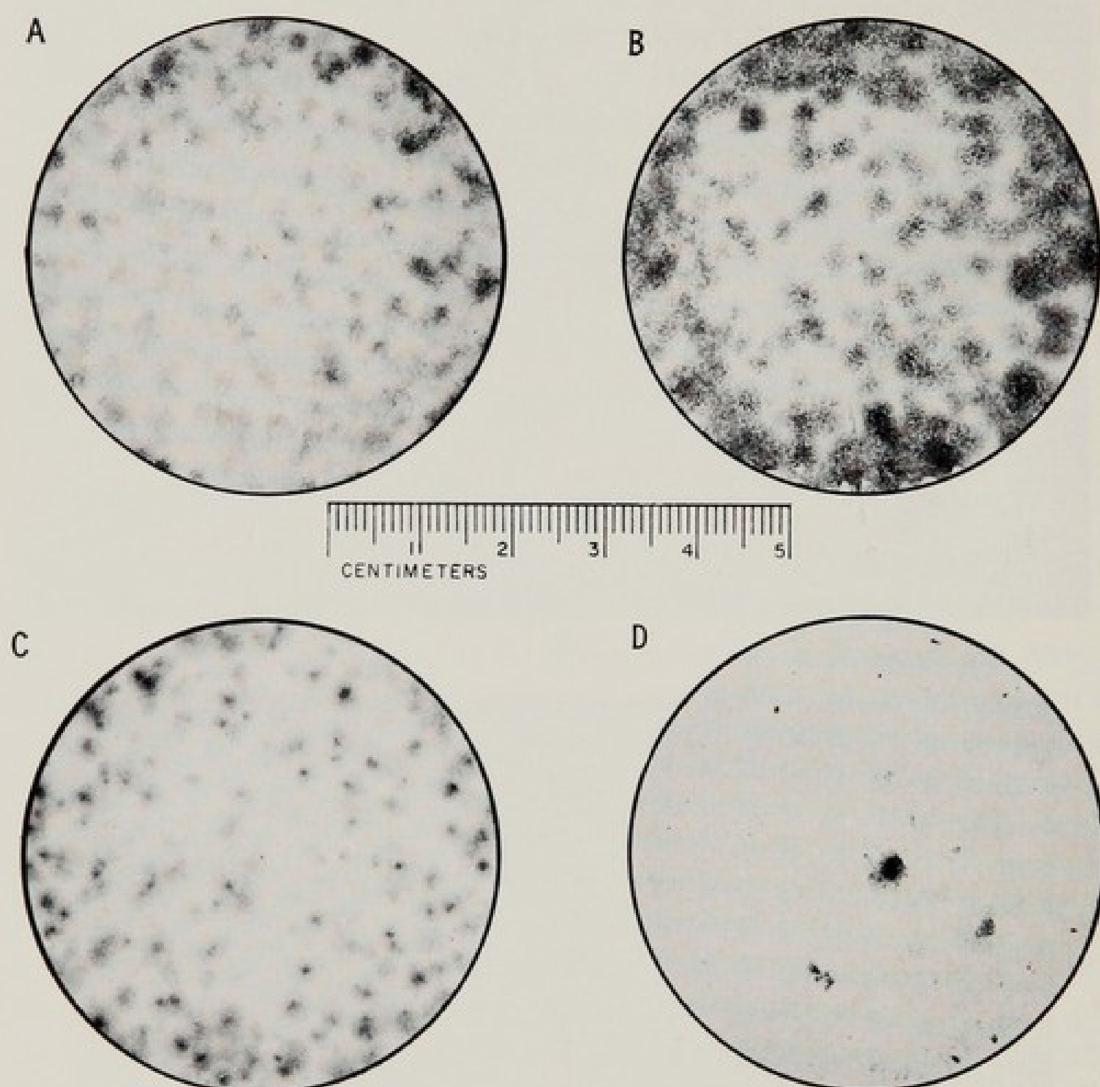


FIG. 9. Second generation fibroblastic clones grown in both conditioned and unconditioned medium. Plates A and B received equal numbers of cells from the same primary colony of fibroblastic cells. Plate A contained conditioned medium and plate B, unconditioned. Approximately the same number of colonies are present in both plates. The same protocol was used for cultures C and D (C, conditioned medium; D, unconditioned). In this case plating was inferior in unconditioned medium.

plates were now inoculated with small numbers of freshly dissociated muscle cells, cultured for a 2-week period, and then examined. Qualitatively, the muscle colonies grown in unconditioned medium but on pretreated petri plates were indistinguishable from colonies grown on conditioned medium (Fig. 10). Pretreatment with unconditioned medium,

however, is completely ineffective. Colonies grown on control plates pretreated with unconditioned medium are in no way different from those cultured in unconditioned medium.

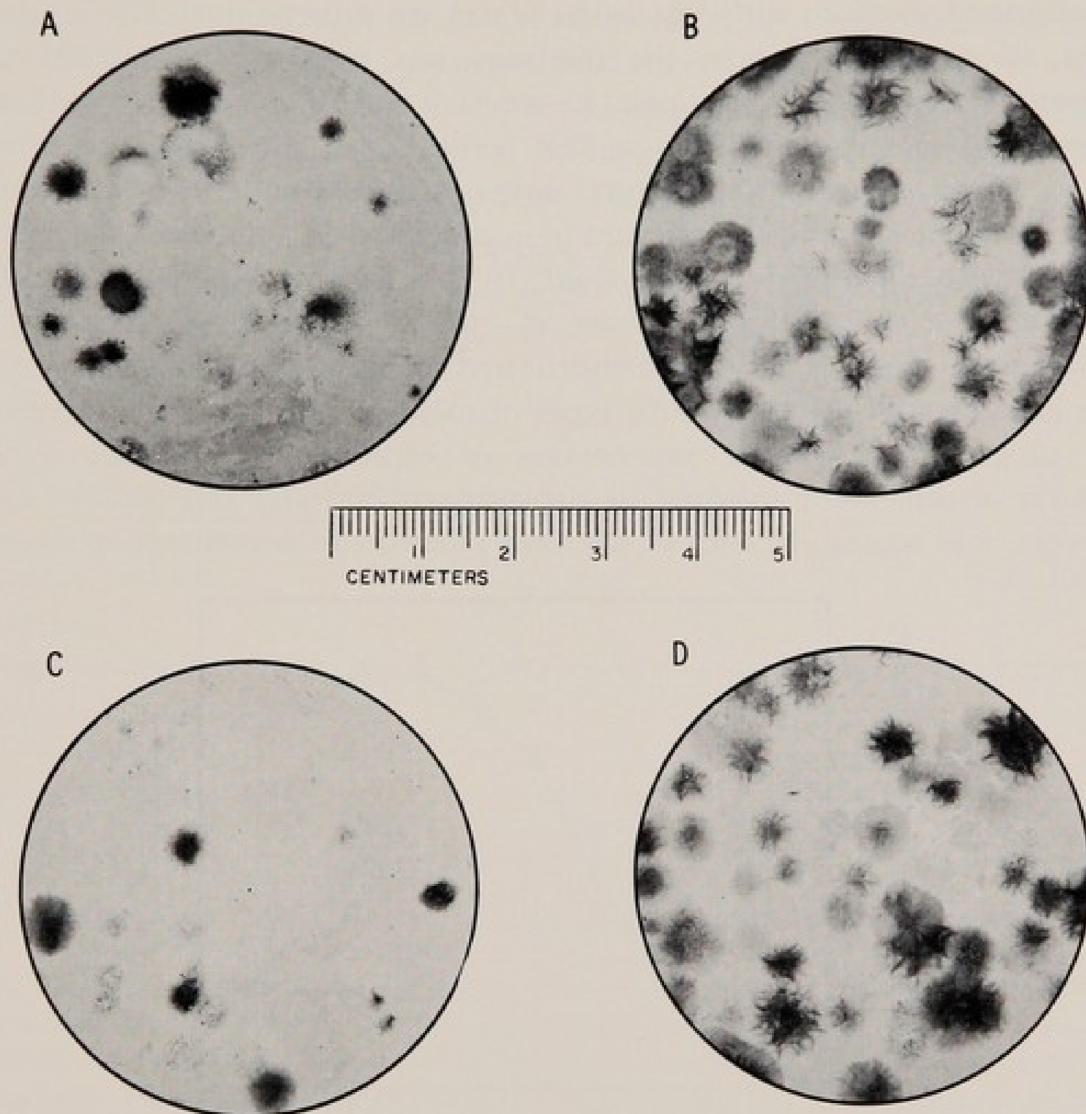


Fig. 10. The effect of pretreating petri plate surfaces with conditioned medium before use. A. Control plate cultured in unconditioned medium. B. Control cultured in conditioned medium. C and D. Cultures grown in unconditioned medium but on pretreated surfaces. The surface of the petri plate in C was treated with unconditioned medium; plate D was treated with conditioned medium. The protocol of pretreatment was as follows: Petri plates were filled with 2 ml of medium and incubated at 36.5°C in a humidified, gassed incubator for 3 days. At the end of this incubation period the medium was removed by aspiration. *All* of the pretreated plates were then filled with 2 ml of unconditioned medium, inoculated with cells, and cultured in the usual manner.

There are three possible explanations for the results obtained by pretreating petri plates with conditioned medium: (1) The petri plate surface is altered by the removal of material; (2) alteration of the surface is

effected by the deposition of material; (3) small traces of soluble material might be left behind on the plates after pretreatment. These small traces may be sufficient to supplement, in the usual nutritional sense, the unconditioned medium with which the plates are subsequently filled.

In all the experiments, the medium was removed by careful and thorough aspiration. Although it seemed unlikely that a significant amount of soluble material was left behind, this possibility was easily tested. If we assumed that the pretreatment results were due to a simple dilution phenomenon, they should be reproduced in conditioned medium diluted with unconditioned medium. In this manner we should be able to determine the minimal amount of conditioned medium required to supplement the volume of unconditioned medium used to test the pretreated plates. Mixtures of both types of medium were prepared containing progressively smaller percentages of conditioned medium. Equal inocula of cells were cultured in these mixtures, fixed and stained after 2 weeks, and scored. In Fig. 11 we have plotted the percentage of muscle

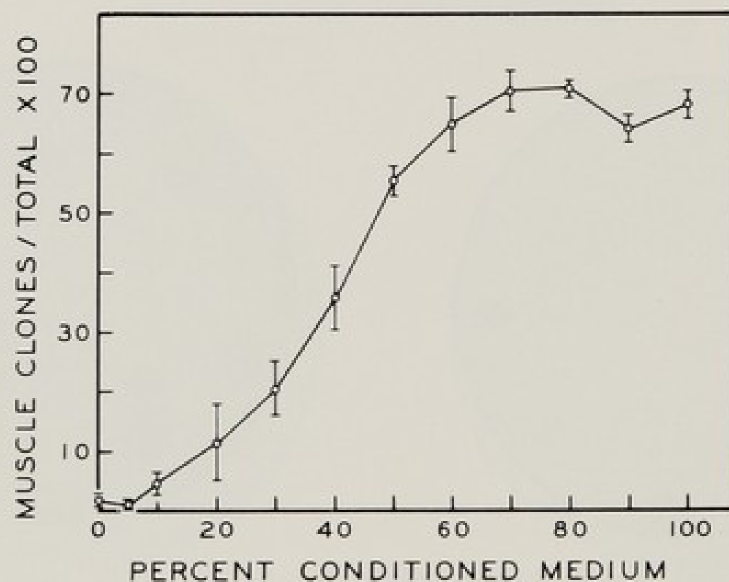


FIG. 11. The percentage of muscle colonies as a function of the percentage of conditioned medium in dilutions of conditioned medium with unconditioned. The standard error of each plotted mean is indicated by the vertical bar through each point.

colonies for each mixture. The percentages of muscle colonies obtained on pretreated plates are included in Table I (see also Table II). Comparing these figures to the values plotted in Fig. 11 we note that the percentages of muscle colonies obtained on pretreated plates correspond to values obtained with mixtures containing 40–50% conditioned medium. Since we pretreat with 2 ml of conditioned medium which is subsequently replaced with 2 ml of unconditioned medium, we would have had to leave

behind almost all the conditioned medium. Such gross carelessness is highly unlikely.

An even more compelling argument than that based on the conditioned medium dilution curve, is the fact that the effect of preincubation persists even after the treated petri plate is rinsed thoroughly with distilled water. Petri plates were again filled with 2 ml of conditioned medium and placed in the incubator for a period of 3 days after which the conditioned medium was removed by aspiration. This time, however, some of the

TABLE I

THE EFFECT OF PRETREATMENT OF PETRI PLATES WITH CONDITIONED MEDIUM UPON THE SUBSEQUENT DEVELOPMENT OF MUSCLE CLONES

Treatment[a]	Number of petri plates in each group	Muscle clones (mean ± S.E.)	Fibroblastic clones (mean ± S.E.)	Per cent muscle (mean ± S.E.)[b]
Unconditioned medium controls	4	0.8 ± 0.5	41.0 ± 3.5	2.0 ± 1.6
Conditioned medium controls	4	28.5 ± 2.1	24.0 ± 1.5	54.3 ± 2.1
Pretreatment (conditioned medium) grown in unconditioned medium	4	18.0 ± 0.7	21.8 ± 2.3	45.8 ± 4.0

[a] Pretreatment as described in text.

[b] Per cent muscle values [(muscle colonies/total colonies) 100] are presented since they normalize the data with respect to intergroup differences in plating efficiency. Tests of the significance of the difference between the means ("Students" t) for per cent muscle indicate that the conditioned medium controls and the pretreated groups are both significantly different from the unconditioned medium controls ($p < 0.001$ in both cases). The mean per cent muscle of the conditioned medium group is not significantly different from the pretreated group ($p > 0.3$). The mean number of muscle clones per plate, however, is significantly different in these two groups ($p < 0.02$).

plates were rinsed either once or three times with 2 ml of sterile distilled water. They were then filled with 2 ml of unconditioned medium, inoculated with a small number of cells, and cultured for the usual 2-week period. Without question, rinsing with distilled water as many as three times gave results indistinguishable from results with preincubated plates which had not been rinsed at all (Fig. 12). In either case the cultures on pretreated petri plates were not significantly different from those obtained on standard plates with conditioned medium. If a film is deposited or adsorbed on the bottom of the petri plate during pretreatment, it must

TABLE II
THE EFFECT OF DISTILLED WATER WASHES UPON THE SURFACE OF PRETREATED PETRI PLATES

Group	Treatment[a]	Number of petri plates in each group	Muscle clones (mean ± S.E.)	Fibroblastic clones (mean ± S.E.)	Per cent muscle (mean ± S.E.)[b]
A	Pretreat: uncond. medium Wash: none Grown: uncond. medium	3	0.0 ± 0.0	38.7 ± 3.3	0.0 ± 0.0
B	Pretreat: cond. medium Wash: none Grown: cond. medium	3	32.3 ± 3.5	22.0 ± 2.7	59.6 ± 2.5
C	Pretreat: cond. medium Wash: none Grown: uncond. medium	3	25.7 ± 2.2	11.7 ± 1.6	68.8 ± 2.6
D	Pretreat: cond. medium Wash: 1 Grown: uncond. medium	3	18.3 ± 1.0	12.0 ± 1.0	60.6 ± 1.7
E	Pretreat: cond. medium Wash: 3 Grown: uncond. medium	3	20.4 ± 2.0	12.0 ± 1.5	63.8 ± 1.2

[a] Pretreatment as described in text.

[b] Per cent muscle values [(muscle colonies/total colonies) 100] are presented since they normalize the data with respect to intergroup differences in plating efficiency. Tests of the significance of the difference between the means ("Student's" t) for per cent muscle indicate no significant difference between the conditioned medium control (B) and any of the pretreated groups (C, D, and E) ($p > .1$ to $p > .8$). The mean number of muscle clones on pretreated plates which were not washed (C) and similar plates washed three times (E) are also not significantly different ($p > 0.1$).

be composed of materials insoluble in distilled water. At present we favor this hypothesis, if for no other reason than that it can be most readily tested. Assuming that some film is deposited on the petri plate surface—what sort of material might it be?

Collagen Synthesis during Conditioning

In reference to our speculations that the fibroblasts were principally responsible for conditioning the medium, one piece of information now assumed importance.

During the period when we were examining alternative protocols for preparing conditioned medium we found that the most effective preparations were those collected from crowded confluent monolayers. Accordingly, farm cultures are grown for 5 days until they reach confluency. At

this time they are refed, and the medium is later collected, filtered, and used. This same protocol is also used when we prepare conditioned medium with farms consisting of second passage cells (predominantly

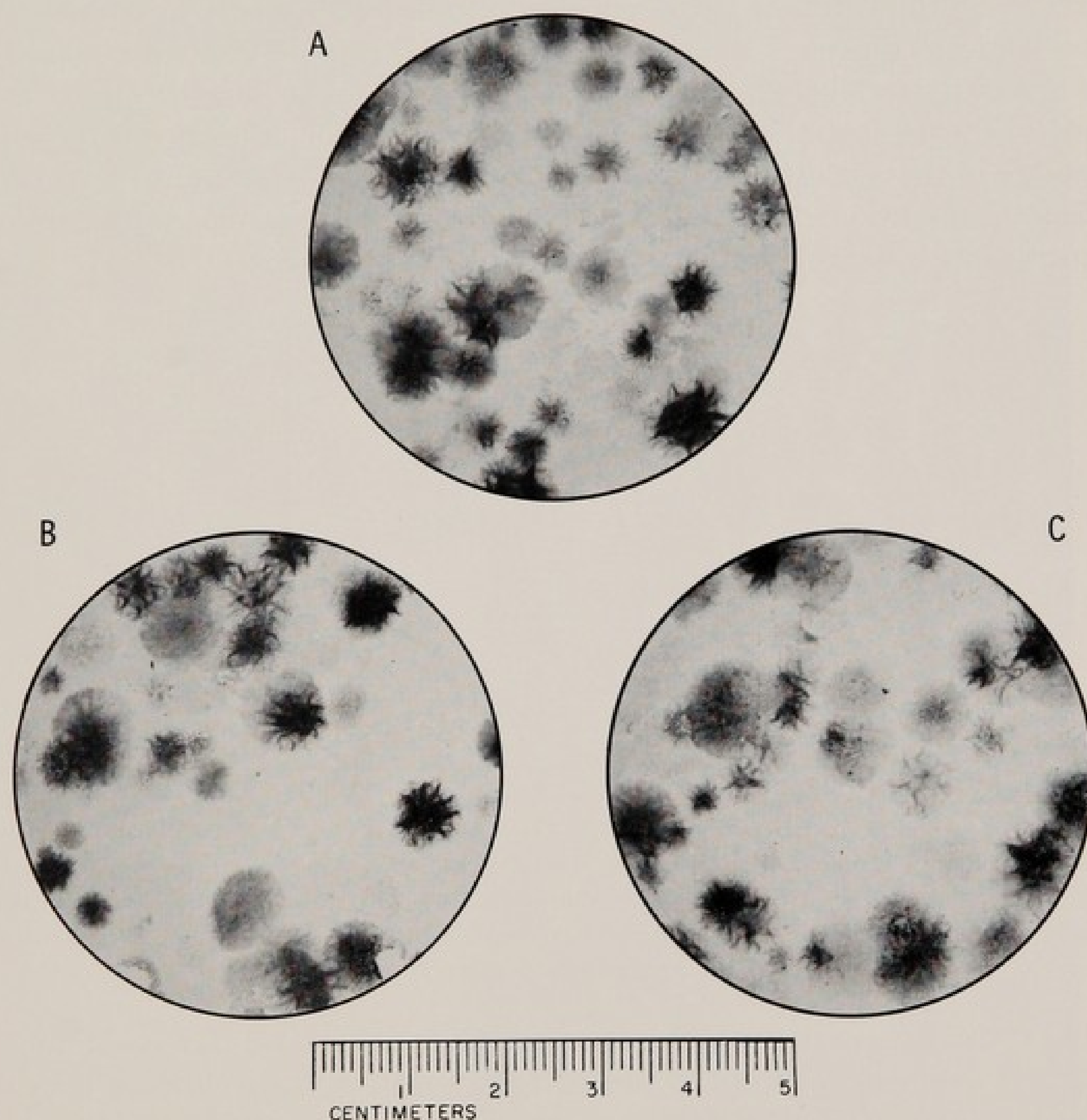


FIG. 12. The effect of washing petri plate surfaces after pretreatment with conditioned medium. The pretreatment schedule is given in the legend of Fig. 10. Cells were cultured on these pretreated plates in unconditioned medium. A. Control: no washes after pretreatment. B. Washed once with 2 ml of sterile distilled water before use. C. Washed with three changes of distilled water, 2 ml each, before use. Each volume of distilled water was left in the petri plate for five minutes and then removed by aspiration. After the last wash the plate was filled with unconditioned medium and inoculated.

fibroblasts). Measurements of the growth of such farm cultures indicate that the cells are in the stationary phase during the conditioning period (Fig. 13).

One striking change in the biosynthetic pattern of fibroblasts which

occurs in the stationary phase has been clearly demonstrated (Green and Goldberg, 1963, 1964; Goldberg and Green, 1964). Using a cell line derived from embryonic mouse cells these investigators have been able to demonstrate both the synthesis and deposition of collagen, but only in stationary nondividing cultures. Using isotopically labeled proline they were unable to demonstrate labeled protein-bound hydroxyproline in either cells or medium from exponentially growing cultures. If our cultures were comparable to those studied by Green and Goldberg, the period during which effective conditioned medium can be collected corresponds to the time during which the cultures should be synthesizing collagen.

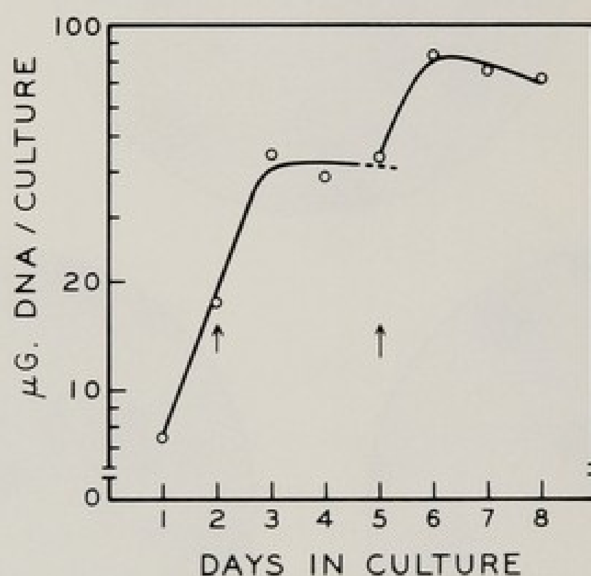


FIG. 13. A semilog plot of the growth, in terms of DNA per culture, of conditioning farms of fibroblastic cells. DNA was determined by Burton's modification of the diphenylamine reaction. The arrows indicate replacement of the medium. Farms were inoculated with 10^6 cells from a suspension of primary cultures.

In order to test the validity of such a comparison, we have examined the cell monolayers in our fibroblast farm cultures using electron microscopic techniques. Cell monolayers were fixed and embedded *in situ* after the conditioned medium was harvested. The results are as yet preliminary, but they indicate that fibrous material is deposited extracellularly between adjacent cells of the monolayer (Fig. 14). This material can be resolved as extremely broad fibers with a definite major period but with no detectable fine periodicity (Fig. 15). Measurements of the interperiod indicate that the periodicity is 850 Å, unlike either native collagen or any of the reconstituted fibers which have as yet been precipitated from solutions of tropocollagen. Such fibers have been observed by Goldberg and Green (1964) in stationary cultures of a mouse fibroblast line. Similar

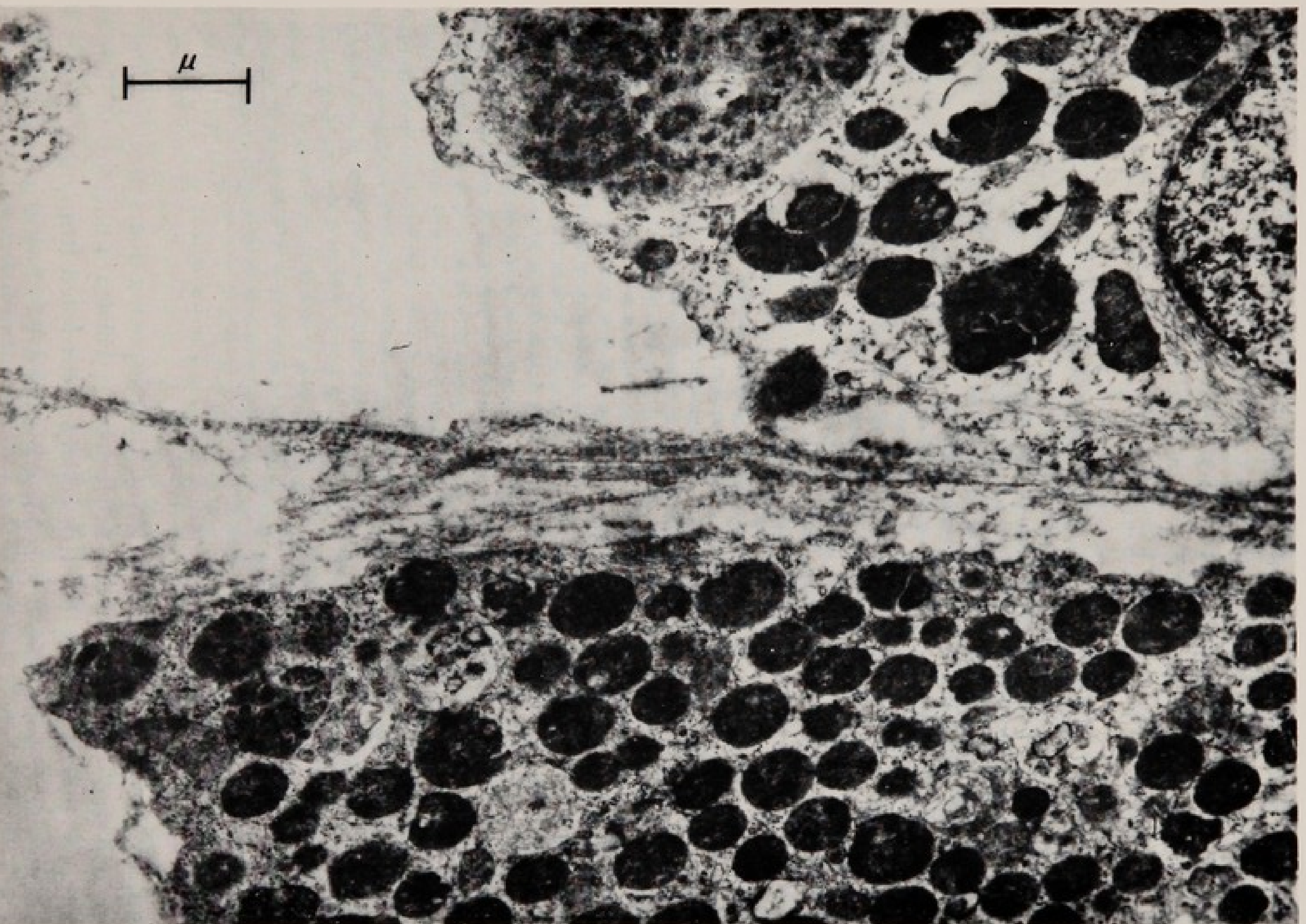


Fig. 14. Electron micrograph of cell monolayer of fibroblast conditioning farm culture. Note presence of cross-banded fibers in the extracellular space between adjacent fibroblasts.

fibers have also been described by Jakus (1961, 1962) in the limbic region of the cornea and most frequently in Descemet's membrane. Jakus (1961) suggests that although no direct evidence exists, the wide variety of fibers observed in the region of the cornea may indicate the existence of an environment in which collagen may assume many forms. She suggests that mucopolysaccharides may be involved in producing these different

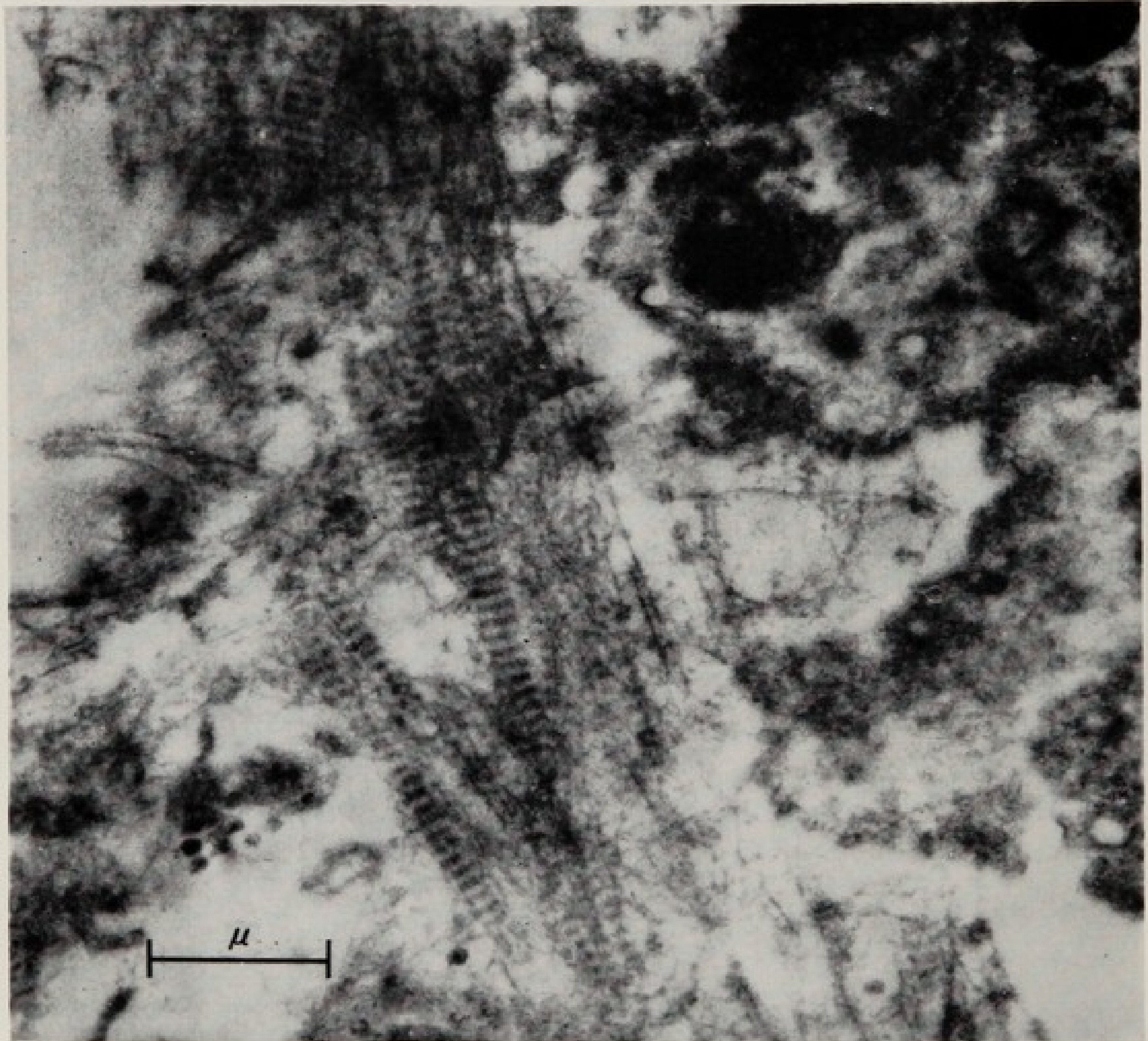


FIG. 15. Fibers similar to those seen in Fig. 14. The periodicity is 850 Å.

forms, and has, in fact, demonstrated such long-spacing fibers precipitating from collagen solutions extracted from the cornea under conditions which give the native spacing with collagens extracted from other tissues (Jakus, 1964). If these fibers are repeatedly dissolved in a citric acid buffer and reprecipitated, eventually only fibers exhibiting the native periodicity are formed (Jakus, 1964). One possible explanation of these observations

is that recycling these fibers removes a contaminant (i.e., mucopolysaccharide) which maintains the long-spaced structure. Our tentative conclusion is that the fibers we have observed in our conditioning farm cultures are an atypical form of collagen.

A Hypothetical Role of Conditioned Medium

The new information which we now had—the facts, as well as the reasonable assumptions can be summarized as follows: (1) Fibroblasts can effectively condition the medium, and perhaps may be solely responsible for conditioning; (2) pretreatment of the petri plates with conditioned medium alters the surface of the plate in some manner so that it will now support the growth and differentiation of muscle clones even when the liquid overlay is unconditioned medium; (3) this alteration of the petri plate surface may be due to the deposition of material on the surface of the plate; (4) in our fibroblast farm cultures, what appears to be an atypical collagen fiber is laid down and we assume that collagen is being synthesized.

Reviewing these points suggests the following hypothesis: Collagen in some form is present in the conditioned medium; it is deposited on the petri plate surface during incubation and there in some manner permits the development of muscle clones from single myoblasts.

If this hypothesis is true, we should be able to extract collagen from a recognized source of the protein and coat the surface of the petri plate with collagen; this tactic should replace the requirement for conditioned medium.

This, in fact, is what we have done. Acid extracts of rat tail tendon were prepared and thin films spread over the surface of the petri plate (Ehrmann and Gey, 1956). Collagen was precipitated either by exposure of the film to ammonia vapor or by adding sodium chloride to 1% of the final volume before spreading the acid collagen film. In either case, after the films had set, they were washed with sterile distilled water and equilibrated against unconditioned medium. Petri plates containing a liquid overlay of unconditioned medium over a surface film of precipitated collagen were inoculated with a small number of single cells. These cultures were subsequently handled in a manner identical to companion controls in either conditioned or unconditioned medium but on conventional polystyrene, tissue-culture petri plates (Falcon).

The results of these experiments were unequivocal. Single cells cultured on collagen films in unconditioned medium gave rise to cultures com-

parable in every respect to controls cultured in conditioned medium. In Table III, the results of six separate trials are tabulated. By quantitative criteria, that is, the percentage of muscle colonies per culture, a collagen substratum completely replaces the necessity for conditioned medium (see Hauschka and Konigsberg, 1966).

In our experience, however, the more reliable and striking characteristic of muscle colonies which develop in conditioned medium is the size and morphology of such colonies. The large sworling muscle colonies, immediately identifiable by their fibrous appearance even to the naked eye, were never before observed in unconditioned medium unless the plates had been pretreated with conditioned medium. They are very much in evidence, however, when single cells are cultured on a collagen film despite the fact that the medium used has not been conditioned (Fig. 16).

Certain experimental facts and reasonable assumptions have led us to construct a hypothetical explanation of the role played by conditioned medium in the development of muscle clones. These same facts and assumptions suggested a simple test of the hypothesis and the results of this test have been overwhelmingly positive. This does not mean that each assumption we have made has been verified. It does suggest, however, that the hypothesis, as a working hypothesis, has merit.

Our thesis is that the development of muscle clones is influenced by the metabolic activity of fibroblasts and that the influence is exerted by collagen or materials associated with collagen. To examine this hypothesis critically, we first need to know if material is indeed deposited on the petri plate during pretreatment with conditioned medium. If this proves to be the case, the identity of these materials will need to be established. Since the amino acid composition and the solubility properties of collagen are so unique (Harrington and von Hippel, 1961), we would anticipate no difficulty in at least establishing whether collagen is or is not one of these materials.

We also need to examine the effects of more highly purified collagens to resolve the question of whether the phenomenon we have observed is due to collagen or to those contaminants usually associated with collagen (mucopolysaccharides, for example). Also of relevance is the question of whether molecules structurally related to collagen would be efficacious.

When we have these answers, we will be in a better position to speculate on mechanisms. We may at least know whether we need consider the uptake of materials into the myoblasts or an effect exerted at the cell surface.

TABLE III

THE EFFECT OF A COLLAGEN SUBSTRATUM UPON THE DEVELOPMENT OF MUSCLE CLONES[a]

Experiment number	Number of petri plates of each type	Conditioned medium (group A)			Unconditioned medium: Untreated surface (group B)			Unconditioned medium: Collagen surface (group C)		
		Mean no. of muscle clones	Mean no. of fibro. clones	Mean per cent muscle	Mean no. of muscle clones	Mean no. of fibro. clones	Mean per cent muscle	Mean no. of muscle clones	Mean no. of fibro. clones	Mean per cent muscle
1	6	38.5 ± 1.1	32.2 ± 2.3	54.8 ± 1.6	1.2 ± 0.4	42.2 ± 3.3	2.6 ± 0.9	31.0 ± 1.1	15.2 ± 0.8	67.3 ± 2.5
2	8	20.8 ± 1.3	28.5 ± 1.4	42.0 ± 0.7	0.13 ± 0.05	34.4 ± 1.2	0.4 ± 0.4	2.19 ± 1.4	14.8 ± 1.4	59.9 ± 3.9
3	6	28.8 ± 2.3	32.7 ± 2.0	48.4 ± 4.0	0.2 ± 0.2	31.5 ± 2.4	0.6 ± 1.8	19.0 ± 0.9	17.0 ± 1.9	53.6 ± 0.9
4	6	20.8 ± 1.9	9.2 ± 0.8	69.4 ± 2.5	0.0 ± 0.0	35.3 ± 2.3	0.0 ± 0.0	20.8 ± 1.3	12.7 ± 0.8	62.2 ± 2.3
5	5	21.6 ± 2.0	10.2 ± 0.4	67.1 ± 2.0	0.0 ± 0.0	27.2 ± 6.7	0.0 ± 0.0	16.6 ± 1.1	17.6 ± 1.5	47.8 ± 5.5
6	6	29.0 ± 2.2	26.2 ± 1.1	52.5 ± 2.0	1.5 ± 0.5	24.2 ± 1.5	6.4 ± 1.9	21.7 ± 0.7	10.3 ± 0.9	67.7 ± 2.3
Average				55.7			1.7			59.8

[a]Values are expressed as means per petri plate ± standard error. Mean per cent muscle values [(muscle colonies/total colonies) 100] are presented since they normalize the data with respect to fluctuations in plating efficiency from experiment to experiment. Tests of the significance of the differences between means for per cent muscle indicate that in every experiment the values for both group A and group C are significantly different from the value of group B ($p < 0.001$ in all cases). Differences between the means of groups A and C were not significant in three experiments and significant or of marginal significance in the other three experiments.

Macromolecules and Cell Culture

The classic approach to the study of the properties of embryonic cells upon which much of modern developmental biology is grounded, in-

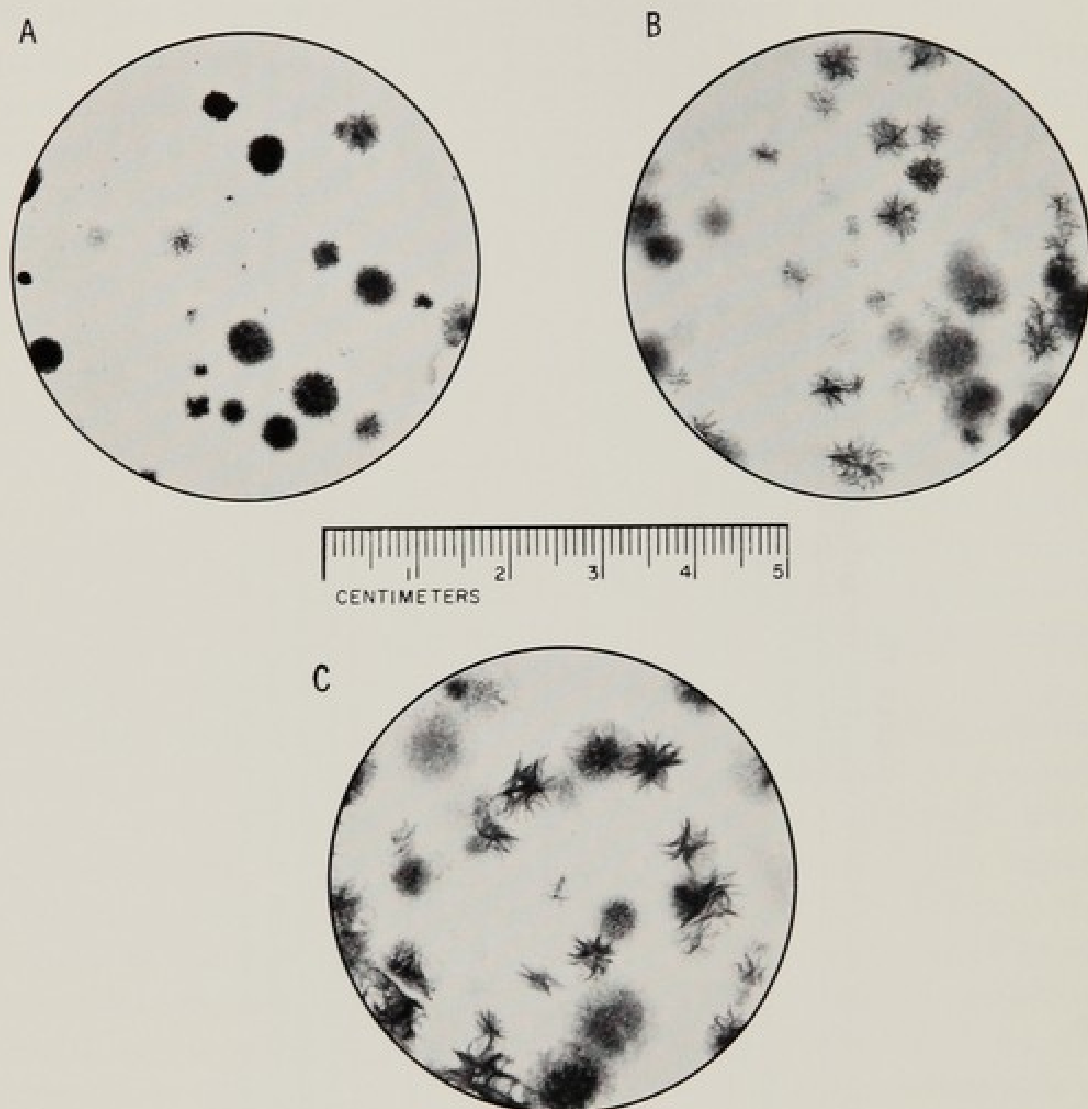


FIG. 16. The development of muscle clones on a substratum of reconstituted collagen in unconditioned medium. A. Control culture: untreated surface; unconditioned medium. B. Control culture: untreated surface; conditioned medium. C. Colonies which have developed on a film of reconstituted collagen with a liquid overlay of unconditioned medium. Compare plates A and C. Both of these cultures have been carried on unconditioned medium. However, note the number of large muscle clones which have developed on the collagen film (C).

volved testing the differentiative expression of a particular group of cells in a variety of ectopic locations in the embryo. Cell and tissue-culture techniques merely extend the range of such tests. In fact, the technique of *in vitro* culture was devised for just this purpose (Harrison, 1907).

Applied intelligently and interpreted with circumspection, culture techniques represent a valuable experimental tool with which to attack the problem of cellular differentiation. It is only when we lose sight of the extremely unusual environmental conditions imposed by culture techniques that we run the risk of overinterpreting our results.

The results we have discussed suggest a dependency mediated via a macromolecular product of one type of cell upon the growth and development of another cell type. We do not as yet know whether the dependency is expressed only when these cells are transferred to the artificial environment peculiar to cell culture.

The role of the macromolecules required by cells in culture has received considerable attention for as long as culture techniques have been employed. Probably the most frequently recurring postulates are either that the macromolecule presents to the cell a required micronutrient or is itself degraded and its subunits reutilized. In fact such functions have been demonstrated in several specific situations (see Harris, 1964, Chapter 5).

Aside from serving a nutritive function, proteins and serum proteins in particular have been implicated in the process of cell attachment to glass. Several attempts have been made to isolate, purify, and identify the serum component or components involved. Indeed, fractions of a high degree of purity have been isolated which possess the cell attachment- and stretching-activity (Lieberman and Ove, 1958; Fisher *et al.,* 1958; Michl, 1962). Since a divalent cation is required for activity, it is assumed that the protein binds the cell to glass through bridging with the cation. Salmine, a basic protein, will also promote attachment and spreading but does not require divalent cations. Moreover salmine-bound cells, unlike those which attach in the presence of the serum factor, cannot be released by ethylenediaminetetraacetic acid. Nor, for that matter, did salmine stimulate growth in the medium employed (Lieberman and Ove, 1959). One might assume that adhesion to glass can occur by more than one mechanism. This premise is further strengthened by the observation that cells, either living or formalin-fixed, adhere even more rapidly and tenaciously to a cleaned glass surface in the complete absence of protein (Easty *et al.,* 1960; Taylor, 1961). Here too, Ca^{++} is not required for attachment, nor can the cells be released by ethylenediaminetetraacetic acid or trypsin (Taylor, 1961; Rosenberg, 1961).

In these experiments the attached cells in protein-free medium neither grew nor survived for any appreciable length of time. Several cell lines, however, have been cultivated in synthetic medium in the absence of any

added macromolecule (see Levintow and Eagle, 1961). Moreover, a defined protein-free medium has been devised which supports single-cell cloning of three lines (Ham, 1965). In this medium the serum protein fraction used to promote cell attachment and flattening has been replaced by the basic amine, putrescine, although other amines are equally effective (Ham, 1964). Whether such relatively simple compounds can substitute for conditioning or for collagen in our system remains to be explored.

We have thus far been considering the relationship of macromolecules only to cell attachment and cell multiplication. There is still too little information on differentiative function in cell culture to do more than pose the question of the possible relationship of maintenance of function to the nature of the substratum. One report is of interest in this context. Hillis and Bang (1959, 1962) demonstrated the outgrowth and maintenance of cells having the morphological appearance of liver parenchymal cells from liver explants, but only when reconstituted collagen gels were employed as a substratum. Control cultures on glass showed either no growth or only some fibroblast outgrowth.

The chemistry of the surface to which cultured cells are apposed has been of interest largely as a tool for studying the chemistry of the cell surface and mechanisms of cell adhesion. Relatively little consideration has been given to the physiological consequences of cell attachment to surfaces of different chemical constitution. In this respect, the work of Rappaport and her associates is unique (Rappaport *et al.,* 1960; Rappaport and Bishop, 1960; Rappaport and Howze, 1964). As a result of more recent studies she has proposed that cell adhesions are mediated through coordination complexing around a monovalent cationic locus. Such a mechanism would require that both the anionic sites on the cell surface and those on the substratum which together form the complex have configurations which satisfy one of the permissable coordination complexes around the cationic locus. Intercellular macromolecules which orient water dipoles and other electrolytes, she suggests, "may be expected to play a significant role" in these reactions. She believes that coordination complexing would permit normal electrolyte transport to occur while maintaining cell adhesion.

Following this line of reasoning, she has been able to maintain several types of cells in environments in which the cation is complexed both by adding high concentrations of ligands of Na^+ and K^+ to the medium and by the use of special glass surfaces. The special glasses appear to bind a larger amount of K^+, the monovalent cation she believes most likely to be involved in normal cell adhesions. Cells maintained for long periods of

time on these glass surfaces in the presence of the ligands still exhibit their cell-type-specific morphology. (Rappaport, 1965a,b; Rappaport and Howze, 1965a,b).

Her proposals are novel but are at variance with the view that divalent cations play a dominant role in cell adhesion (see Steinberg, 1958, 1962). We still know too little about the physiology of cell adhesion, however, to warrant dismissal of suggestions which do not seem to fit into the flimsy framework of our present knowledge.

Collagen and Differentiation *in Vivo*

Those of us who employ cell and tissue culture techniques for studying developmental phenomena *in vitro* are continuously forced to evaluate and re-examine our results from the vantage point of normal developmental events occurring in the animal. Are the phenomena we study in culture related to the events occurring in the organism? Our present data indicate that the development of a muscle clone in culture requires the presence of a metabolic product of another type of cell, the fibroblast. Does such a dependency exist in developing muscle tissue *in vivo?* We know of no concrete evidence that such a relationship does exist, nor would such an interaction have been anticipated or suspected.

It would not have been suspected because the morphological clues which have led embryologists to anticipate the existence of such interactions are not present in developing muscle tissue.

There are a multitude of tissue interactions of the type classified as embryonic inductions. All these interactions occur between two groups of cells which, although they may be in intimate contact, have a sharply defined boundary delimiting them. A dramatic morphological or cytological change within tissues so arranged suggested the appropriate separation and recombination experiments which established the dependency (Spemann, 1938).

The optic vesicle-lens induction system, in which these morphological clues are so dramatic, was the first to be experimentally established. In contrast to these histologically "tidy" systems the two cell types in muscle which we suspect of being components of an interacting system are more or less randomly distributed throughout the tissue. We are therefore in the position of asking whether this phenomenon, which could only have been discovered with cell culture techniques and more specifically with cloning techniques, has any relevance to the *in vivo* realities.

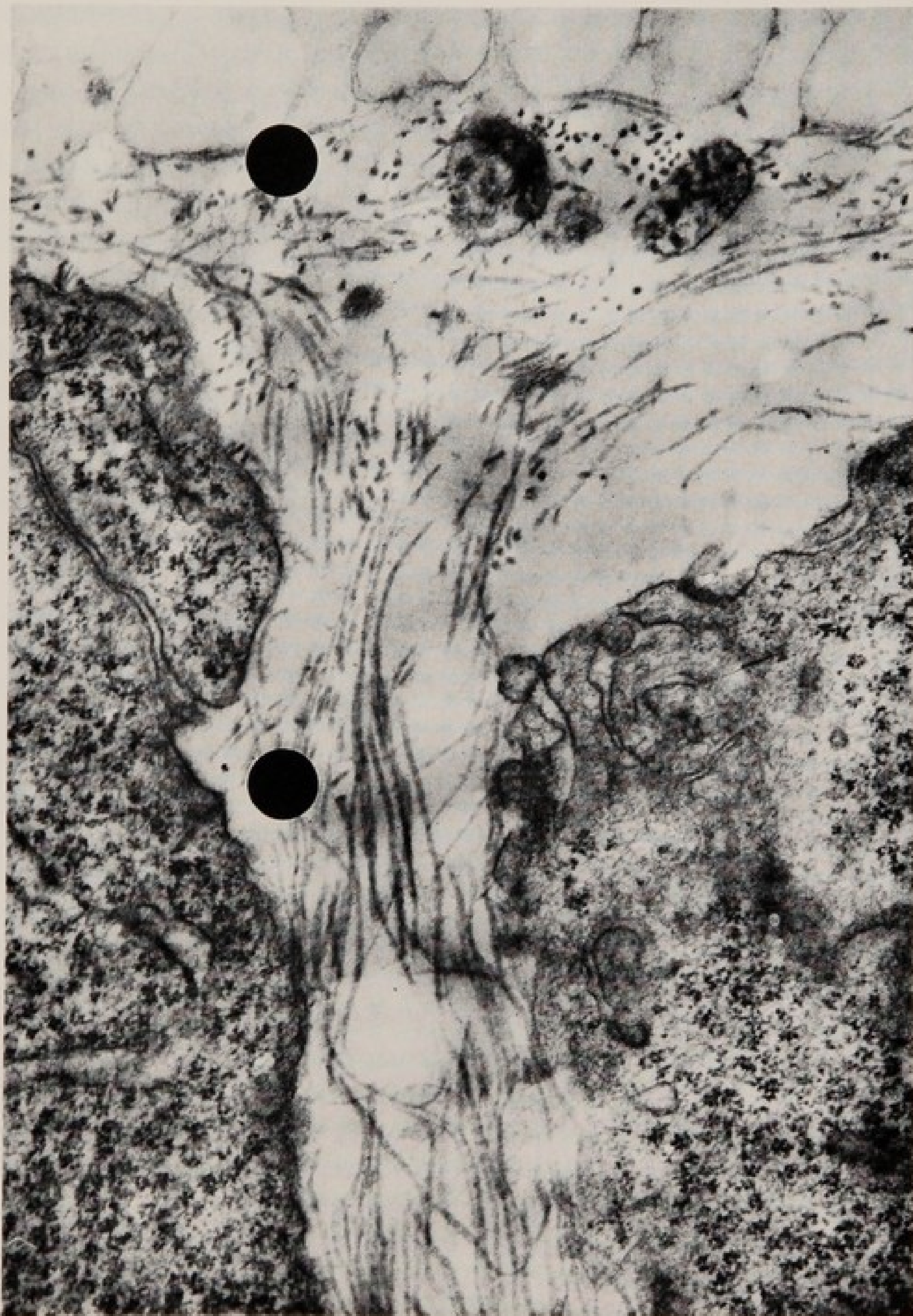
Although the particular interaction we have observed in culture has

not been recognized *in vivo*, the initiating cell type, the fibroblast (or its progenitor, the mesenchymal cell) participates in a host of well-established embryonic inductions. In all these interactions the responding tissue is a well-ordered epithelium which again can be clearly delineated from the mesenchymal component.

By recombining the epithelial component with mesenchyme from a variety of sources it has been possible to examine the tissue specificity of interaction. Of the epitheliomesenchymal interactions studied thus far (Table I, Rutter *et al.*, 1964) only kidney and salivary gland epithelia show a highly specific requirement for mesenchyme from the same organ rudiment (Grobstein, 1954). In contrast, differentiation of the epithelium of both thymus (Auerbach, 1960) and pancreas (Golosow and Grobstein, 1962) can occur under the influence of mesenchyme from a variety of sources. Although tested in only a small number of experimental recombinations the differentiation of the epithelial components of two other organs, pituitary (Sobel, 1958) and lung (Dameron, 1961), will occur under the influence of mesenchyme from at least one source other than the homologous organ. This suggests that in the less fastidious cases, at least, some metabolic activity common to fibroblasts generally may be responsible for the morphogenetic effect. The one universal activity of the fibroblast irrespective of its location is the synthesis of tropocollagen. We might raise the question whether collagen production is in fact the common denominator of most, if not all, of these interactions. Although the experimental evidence is meager, a few studies contain relevant information.

By far the most provocative observations have been made by Kallman and Grobstein (1964) employing the transfilter technique to study tissue interactions in the developing pancreas. An earlier study (Grobstein, 1962b) established that the critical period with respect to the persistence of acinar cell differentiation was between 24 and 48 hours after the culture was established. After this time, even if the mesenchymal component was removed, the epithelium continued its differentiation. Examining the cytological fine structure of these transfilter cultures, Kallman and Grobstein (1964) found that two significant events occur during the "critical

FIG. 17. Electron micrograph of pancreatic epithelial cells at the interface of the epithelium and the Millipore filter after 4 days in culture. Fibers exhibiting a periodicity of approximately 600 Å, appropriate for collagen, are observed at the filter surface and also extending up between the basal surfaces of the two adjacent epithelial cells. Black spheres represent 260 mμ polystyrene latex particles. (From Kallman and Grobstein, 1964, p. 407.) (Photomicrograph courtesy Drs. Kallman and Grobstein.)



period." There is an increase in the number and structural organization of ribosomes in the epithelial cells; and at the basal surface of the epithelial cells, fibers can be observed in which eventually a periodicity consistent with that of collagen can be recognized (Fig. 17). Similar fibers are also seen in the inductive interaction between salivary gland epithelium and mesenchyme in transfilter culture. More recently these investigators have extended these observations with a radioautographic analysis of protein synthesis and transport across the filter (Kallman and Grobstein, 1965). Their observations are consistent with the thesis that soluble collagen is synthesized in the mesenchymal cells, diffuses across the filter, and then aggregates at the cell boundary of the epithelial cells. Certainly it would be imprudent to infer from these observations that the fibers play any active role in the interaction. They may instead represent the end product of such an interaction. These epitheliomesenchymal interacting systems, however, would seem to lend themselves very well to the type of analysis needed to examine the possible role of collagen fibers in these interactions. Unfortunately, only two such studies have been reported.

In both studies the epidermal-dermal relationship was investigated. Dodson (1963) used scale-forming skin from the tarsometatarsal region of the 12-day-old chick embryo. The separated epidermis, he found, which cannot be maintained when cultured separately, survives and differentiates when cultured in contact with frozen-thawed dermis despite the absence of viable dermal cells. Since collagen represents the major extracellular component of the dermis he also cultured isolated epidermis on a layer of collagen gel. This too he found would support differentiation. Wessells (1964b), studying isolated epidermis from the same region, used the maintenance of proliferating cuboidal basal cells to assess the adequacy of various mechanical substrata to replace normal dermis. He confirmed Dodson's finding that both frozen-thawed dermis and collagen would replace living dermis. These investigations are in conflict only on one point. Although Dodson found that isolated epidermis could not be maintained on a support consisting simply of a Millipore filter, Wessells obtained positive results using such membranes. He found, however, that none of the substrata which he employed was effective unless the nutrient medium contained either a high concentration of embryo extract or a particulate fraction of the extract. Whenever material as poorly defined as embryo extract is required, the reservation must always be made that

a multiplicity of requirements is being satisfied. For example, the extract may be supplying a nutrient, without which the cell cannot respond to the presence of the collagen gel, rather than replacing the gel. Although Wessells considers the two requirements for a continuous solid substratum and a particulate fraction separate and nonsynergistic there may, of course, be some complementarity.

In an earlier report Rutter, Wessells, and Grobstein (1964) described some of the properties of the particulate fraction. The activity distributes itself in a heterogeneous fashion within the sedimentable fractions. A heterogeneous mixture of collagen fibrils of different degrees of aggregation would behave similarly. DNase and RNase do not affect the activity of the particulate fraction but trypsin does. The authors suggest that protein may play a dominant role. They also suggest that "the differentiative activity may reside in the intracellular matrix or may be bound to the cell membrane." As Rutter and co-workers point out, however, we can resolve questions of a possible multiplicity of factors, the identity of the particulate fraction factor, and mesenchymal cell activity only by isolating and characterizing the active substance or substances.

The speculation that extracellular materials play a directive role in developmental events has received particular attention during the past decade (Grobstein, 1954, 1962b). In considering such a hypothesis our attention is usually drawn to the mucopolysaccharides rather than to collagen. We have by no means excluded, as yet, the possibility that the results obtained by cloning on collagen films are due to mucopolysaccharide intimately associated with the collagen (or for that matter to other contaminants). The test system we are using, however, should greatly simplify an examination of this sort. Until we have explored the question in the laboratory it does not seem profitable to pursue the question at length on paper. It may be of interest, however, to examine one line of reasoning which might make the mucopolysaccharides more attractive than collagen as possible mediators of differentiative phenomena. By virtue of their great variety, complexity, and high specificity one could more readily envision this class of macromolecules influencing a wider spectrum of diverse differentiative events. As we pointed out earlier, however, many of the epitheliomesenchymal interactions known to us do not show a high degree of specificity, and the epithelial component will respond to mesenchyme from a variety of sources. It may be somewhat gratuitous then, to insist upon specificity at the molecular level. The dif-

ficulty, perhaps, is that we tend to view all inductive events as requiring that the responding cell receive and take up some product of the effector cell which then plays some determining role in the emerging metabolic pattern (but see Grobstein, 1962b, 1963). This may occur in some inductions, perhaps in all. Yet in the absence of any compelling evidence it might be interesting to consider other possibilities. For example, no exchange may in fact occur, but the interaction may enable the cell to retain metabolic products which it can synthesize but which it would otherwise lose.

There is no reason to assume that all cell and tissue interactions share a common mechanism. The regulation of cellular differentiation may not invariably require the input of information. Current thinking suggests that cellular differentiation is the expression of differential readout of a store of information common to all cells. Among the factors which may maintain or elicit differential gene expression we must consider those mechanisms which promote the retention of information, in the broadest sense of the term, within the cell.

Summary

The reproduction of specialized types of cells during embryonic and adult life occurs in two distinctly separate phases. Cell reproduction takes place in a population of relatively unspecialized cells. The overt acquisition of specialized structure and function, however, occurs in nonproliferating tissue elements. This dichotomy is most readily appreciated in tissues in which the reproductive cells are segregated into a histologically separate stem cell layer.

It had been suggested that the regulation of differentiation in stem cell populations is mediated by a differential division of a stem cell into two daughter cells, one of which retains stem cell properties, the other destined to acquire specialized function. However, such a hypothesis is not consistent with the observed subsequent behavior of the products of individual stem cell divisions.

A common pattern in many segregated stem cell populations is that subsequent specialization is associated with emigration out of the proliferative zone. Cytodifferentiation, therefore, may be a response to the new cellular environment of the migrating cell. The directive role of cell and tissue interactions in development may not be restricted to embryo-

genesis. The bulk of our information about such interactions, however, comes from studies of embryonic tissues.

Studies of embryonic induction, particularly those inductive events occurring in mid-embryonic life, have within the past decade altered our concepts of the regulatory function of cell and tissue interactions in cellular differentiation. The most important contribution has been the demonstration that these interactions do not require direct cell-to-cell contact.

In the belief that it offers a unique opportunity to study cell interactions in a differentiating system we have continued an earlier investigation of the development of clones of differentiated muscle from isolated embryonic myoblasts. The development of such clones is wholly dependent upon the use of conditioned medium (medium which has been exposed, for a time, to the metabolic activities of a large population of cells). For all practical purposes, the multiplicity of possible alterations to medium conditioned in this fashion precluded a conventional biochemical analysis.

Instead we elected to define the biological parameters of the activity of conditioned medium, hoping to be able to narrow the search for the effective alterations of the medium.

These investigations suggested that: (1) Fibroblasts could condition the medium and may be solely responsible for conditioning; (2) pretreatment of petri plates with conditioned medium alters the surface, perhaps by the deposition of material, so that it can support the development of muscle clones in unconditioned medium; (3) the synthesis of collagen in conditioning cultures of fibroblasts is suggested by the presence of what appears to be an atypical collagen fiber.

These observations led to the hypothesis that some form of collagen is present in conditioned medium and is deposited on the petri plate surface during incubation where it permits the development of muscle clones from single myoblasts.

To test this hypothesis a thin film of collagen obtained by acid extraction of rat tail tendon was precipitated on the surfaces of petri plates which were then used to culture embryonic cells with a liquid overlay of unconditioned medium. The collagen substratum completely replaced the requirement for conditioned medium.

Although not specifically demonstrated in the case of myogenesis, the

participation of mesenchymal cells in a variety of inductive events has been described. In one such case, the differentiation of pancreatic epithelium, the deposition of collagen fibers occurs during the same period that the mesenchymal component has been shown to exert its directive influence. Collagen or materials associated with it may play a common role in the regulation of many diverse differentiative events.

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Aging As a Consequence of Growth Cessation

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Introduction

In comparison with many other areas of biology, useful concepts regarding aging processes have been evolving very slowly. While much information has accumulated, there is little agreement concerning the significance of various observations. Few of the most active workers in this field would agree on the specific questions to be asked and the types of experiments to be performed to obtain a better understanding of the mechanisms of aging. The slow progress in aging research has been partly due to oversimplifications, advancing of general theories which allegedly explained diverse and probably unrelated phenomena, and confusion in regard to the kinds of processes to be categorized under the unsatisfactory term of "aging." Progress has also been slow because of difficulties inherent in the study of aging processes: the frequent necessity for maintaining organisms for long periods, difficulties in distinguishing between diseases and aging, and the lack of adequate control systems, i.e., systems existing in time but not undergoing change. In addition, perhaps many of the most competent biologists have ignored aging processes because on superficial examination these processes do not appear to contain the programming and certainty so attractive to most investigators.

In this discussion, we will develop the thesis that enough information is available to classify and make clear distinctions between different types of aging processes and that for some types we know enough to construct conceptual frameworks which can tell us what questions to ask and what kinds of investigations would be most informative. In association with this thesis, several arguments will be advanced: Aging processes, which are widespread in nature, should be considered "normal" biology and as such are worth studying and understanding; aging is closely related to growth cessation, but only when certain cell or tissue constituents are retained; a primary distinction should be made between aging of an intact metazoan and aging of an organism's cells and tissues. For example, almost

all of the cells a hydra has today will have aged and died in 6 weeks but the hydra itself will still be vigorous at that time (Burnett, 1961). On the other hand, if we study a very old human being who is about to die, we will find that essentially all of his cells appear normal by metabolic and chemical criteria. Some questions provoked by these observations are: What is the mechanism of cell death in hydra; why does the human being die when his cells appear viable; and, what cell or tissue changes could explain the age-related death in a complex higher animal? In this discussion it will be argued that the common denominator in cell and animal aging is growth cessation, that mechanisms in the two cases are quite unrelated, and that enough is known, particularly about aging of some higher animals, for us to propose plausible descriptions of these mechanisms.

Scope of Aging

An aging process can be usefully defined as one occurring in all members of a population and which has its onset, or progresses more rapidly, after maturity. Maturity, therefore, is the border between traditional developmental biology and gerontology. Aging processes progress and are not reversible under usual physiological conditions. Progression also distinguishes aging processes from other biological reactions which either stop or are maintained at equilibria by feedback mechanisms. Reactions which progress are generally harmful to the system involved, since homeostasis depends on the establishment of equilibria or steady states. Such progression in biological systems frequently results in death; and death is therefore a convenient endpoint in the study of aging processes. In a human context, it can be argued that aging processes are responsible for the only 100% fatal disease everyone has. Biological processes which constitute a barrier to human immortality should be of more than casual interest.

Aging processes are exceedingly widespread, both in nonliving systems and in living systems which have attained certain levels of complexity and organization. Among the nonliving systems, almost all man-made materials and machines age, some at rates and by mechanisms apparently analogous to the aging of higher animals. Conspicuous aging occurs in plastics, paper, and rubber. It is perhaps significant that these materials are high polymers, as are substances in higher animals which show prominent age-related changes. In living systems aging processes as defined above occur at all levels from the macromolecule to the intact mammal.

In many cases rates of aging and occurrence of death endpoints can be predicted and described with the same precision as developmental stages, growth rates, and metabolic reactions.

Bacteria in culture age, die, and lyse in a predictable fashion. In some species bacterial aging has been associated with characteristic changes in morphology and cell wall composition (Chatterjee and Williams, 1962; Collins, 1964). Among the protozoa, *Tocophrya infusorium* degenerates and dies by aging processes (Rudzinska, 1951). Similarly, gametes which do not participate in fertilization pass through a sequence of degenerative processes ending in death. In tissue culture, under optimal conditions of serial passage, diploid cell strains die out in 4 to 10 months (Hayflick and Moorhead, 1961; Miles, 1964). Hayflick and Moorhead (1961) favor the hypothesis that an intracellular factor necessary for survival is not replicated as fast as the cells are replicated. It is not likely that such rapid and continuous cell division occurs in nature, and this example of aging processes in proliferating cells may not provide information about other aging systems.

In metazoans a distinction must be made between aging of the intact organism and aging of the various cell populations within the organism. While both aging phenomena satisfy the criteria of aging processes, they proceed independently of each other and appear to involve different mechanisms. Aging of intact animals, characterized by clearly defined loss of function, degenerative structural changes, and a specific type of age-related mortality rate, is seen in most phyla. It is most conspicuous in animals showing both a species-specific size and maintenance of stable tissues in the adult. Such aging has been studied most thoroughly in insects, laboratory mammals, and man; it will be dealt with in some detail below.

Many cell populations in both embryos and adult animals undergo an apparently clearly programmed senescence. In development, cellular degeneration and death are noted in such instances as the involution of ascidian and anuran larval tails, loss of gills, involution of insect tissues during metamorphosis, disintegration of chrondrocytes in osteogenesis and involution of Müllerian and Wolffian ducts in male and female mammals respectively. An orderly sequence of cellular degenerations occurs in tissue invagination and separation, in the formation of lumina, and in tissue and organ modeling. In most of these cases cell degeneration and death are probably essential for subsequent normal development. Such degenerations are programmed to the extent that transplantation experiments can be performed with presumptive degenerative tissue.

Some types of developmental degeneration appear to result from intrinsic cellular factors while others are influenced by hormones or other tissues (Biggers, 1964; Zwilling, 1964). After organ systems have developed and throughout the life of an animal, many cell populations demonstrate phases of cell division, cell specialization, degeneration, and death. The last two mentioned phases satisfy the criteria of aging processes and will be considered in some detail.

Growth and Aging

From a priori considerations, we would predict that aging processes would be more conspicuous after growth cessation. For a system to change with time, either different components must be added or subtracted at different times, or there must be changes in components which remain. Substances which are constantly turning over and being replaced would not be expected to contribute to an aging process. Similarly, a growing system is constantly adding to itself new (and young) components which, even though added to a cell or tissue present for some time, would not contribute to aging until present long enough to undergo change themselves. It follows that the aging rate of organelle, cell, tissue, or intact organism would be proportional to the amount of biologically important nonrenewable materials present and the time during which such materials are present. The aging rate would of course be a manifestation of the changes occurring in these materials which alter their biological properties.

Accumulated data have supported the notion that aging characteristically follows growth cessation. In nature, cell populations which degenerate on schedule and show an age-related increase in death rate are those which stop dividing and which presumably contain some stable elements. The metazoans which demonstrate the most exact life-spans and sequence of degenerations are those which attain a size characteristic of the species and grow no more, and which retain significant amounts of nonrenewable body constituents. It has been noted that senescence is the necessary price paid for evolutionary attainment and maintenance of an ideal size in higher animals (for discussions of evolution and aging, see Strehler, 1962, and Comfort, 1956). Experimentally, it has been possible to lengthen the lifespan of mammals considerably by prolonging the growth phase (McCay, 1952). Factors causing cessation of growth appear to represent the last interest of traditional developmental biology and the first interest of gerontology, although neither growth nor aging can be

appreciated without some understanding of their interrelationships. The remainder of this discussion will be devoted to examples of the two most conspicuous types of aging in nature: aging of intact higher animals, and aging of certain cell populations within metazoans.

Animal Aging

From a consideration of the relationship between growth and aging, it follows that aging of higher animals can best be studied in those with a species-specific size. Human beings satisfy this criterion and will serve as examples in much of the following discussion. Humans are also valuable in considerations of aging because so much is known about alterations in their physiological processes with time, age-related changes in morphology, chemical composition, disease incidence, and mechanisms of death and death rates of large populations. For the construction of hypotheses explaining aging mechanisms, it is essential that something be known about the factors which precede the death endpoint. This is particularly true when lifespans are altered by some experimental procedure or when genetic variations in life-span are under study. Too frequently differences in lifespan alone resulting from genetic variation or special treatment, are used in generalizations about aging. Such differences may be due in some cases to changed patterns of disease or specific lesions which have little relationship to aging in a natural population.

Some Characteristics of Aging Populations

If death is considered the endpoint of human aging, some inferences regarding mechanisms can be drawn from the inspection of death rates of different populations plotted in different ways. If the percentage surviving is plotted as a function of age, different types of curves are obtained from different populations. In Fig. 1, the death rate of the British India population yields a curve somewhere between the logarithmic die-away curve expected from random nonage-related deaths and the more rectangular curve for the New Zealand population. In general, the more highly developed societies show the most rectangular types of curves. This results largely from the reduction of deaths due to infectious diseases which either affect a young population or show a poor correlation with age, allowing the population to die from age-related processes. The curve for the New Zealand population demonstrates very clearly the age-related increase in the probability of dying. Such rectangular curves, indicating an increasing death rate with increasing age, are characteristic of popu-

lations having members that wear out, such as a population of automobiles. The various curves in Fig. 1, in spite of their shapes, all end at approximately the same place on the age axis, indicating that the maximum life-span is about the same in the different populations. It may be inferred from these curves that continuing progress in sanitation and immunity programs and in treatment of disease will result in survival curves becoming more rectangular, but will not lengthen the human life-span.

Information concerning reasons for the dying out of human populations can be obtained by referring to accepted causes of death. Some reser-

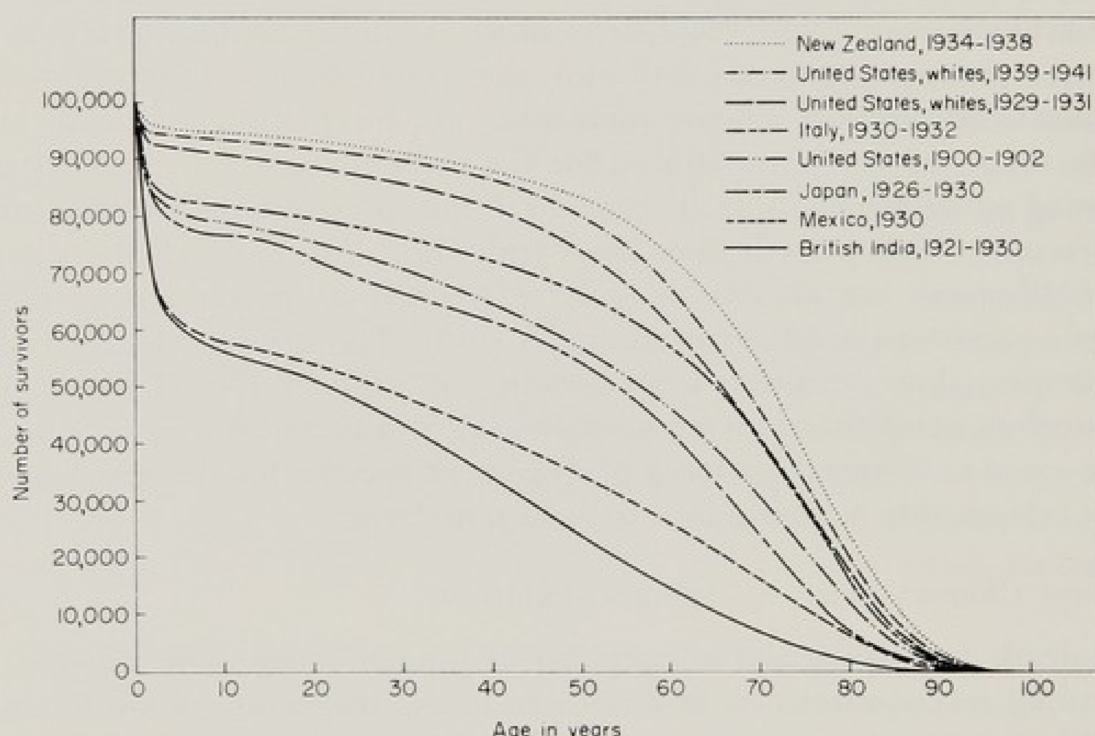

FIG. 1. The number of survivors out of 100,000 male live births; from life tables for selected countries (Comfort, 1956).

vations are required in interpreting these data since in many individuals different diseases progress concurrently and it may be fortuitous that one disease appears to kill rather than another. It is also likely that the effects of one disease may be altered by the presence of another. In Fig. 2 the age-specific deaths for all causes yield a straight line when plotted logarithmically as a function of age after maturity. This type of age-specific death rate is an important feature of an aging population (Gompertz, 1825). It indicates that in a human population the rate and probability of dying double about every 8 years after maturity. Curves for the main causes of death in Fig. 2 include some which parallel or rise faster than the curve

for all causes indicating a very strong age-dependence for deaths due to the disease involved, and some which rise more slowly or drop off with increasing age indicating less of a relationship to age. Most deaths are due to specific lesions such as varieties of arteriosclerosis, malignancy, and hypertension. It can be shown, however, that if these diseases did not exist or were all cured, mean life expectancy would increase very little

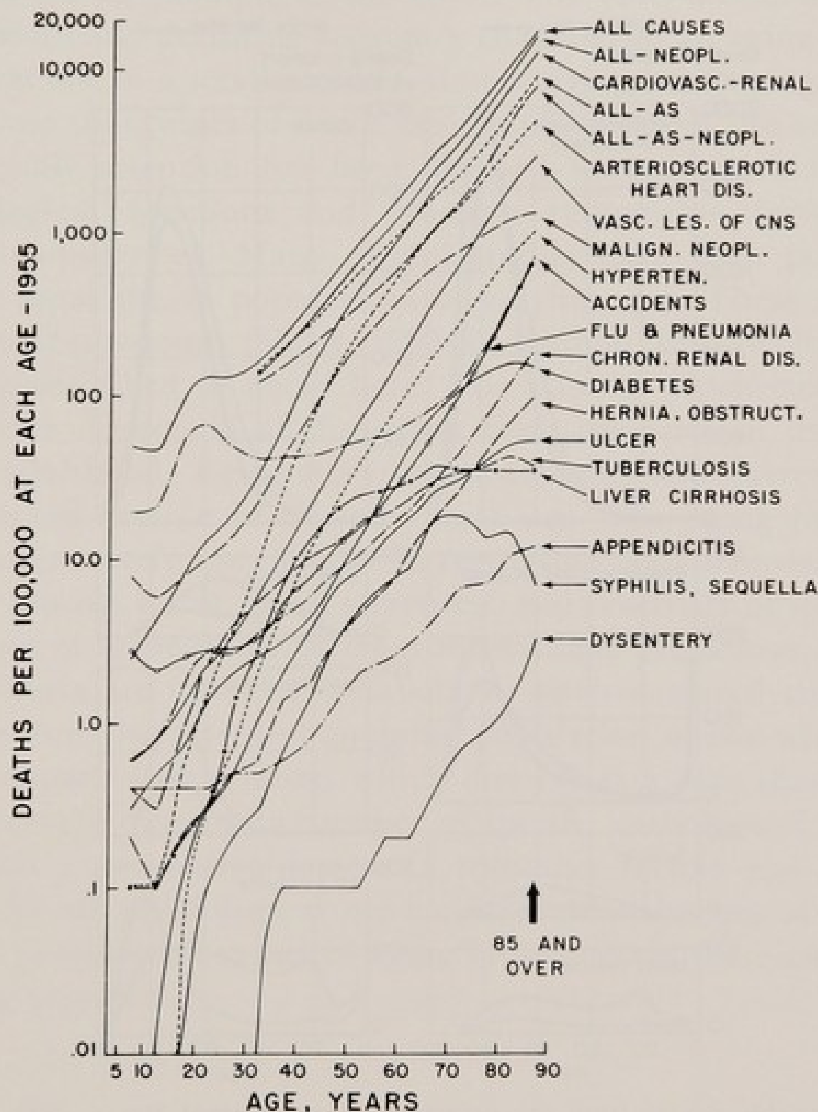

FIG. 2. Mortality from selected causes by age (Kohn, 1963).

and maximum life-span probably not at all. A population without cancer would have its curve for all causes shifted to the right as indicated in Fig. 2 (curve ALL-NEOPL.), increasing the life expectancy of the population 1–3 years. Similarly, conquest of arteriosclerosis would increase life expectancy about 7 years (curve ALL-AS). Absence of both neoplasms and arteriosclerosis would not add more than 10 years to life expectancy

(curve ALL-AS-NEOPL.). The population would then die off from a large number of pathological processes, headed by respiratory infections and accidents. In later decades of life the curves for influenza and pneumonia and for accidents rise more rapidly than the all causes curve (Fig. 2) and these processes would be largely responsible for maintaining the present maximum life-span. This can be seen more clearly when fre-

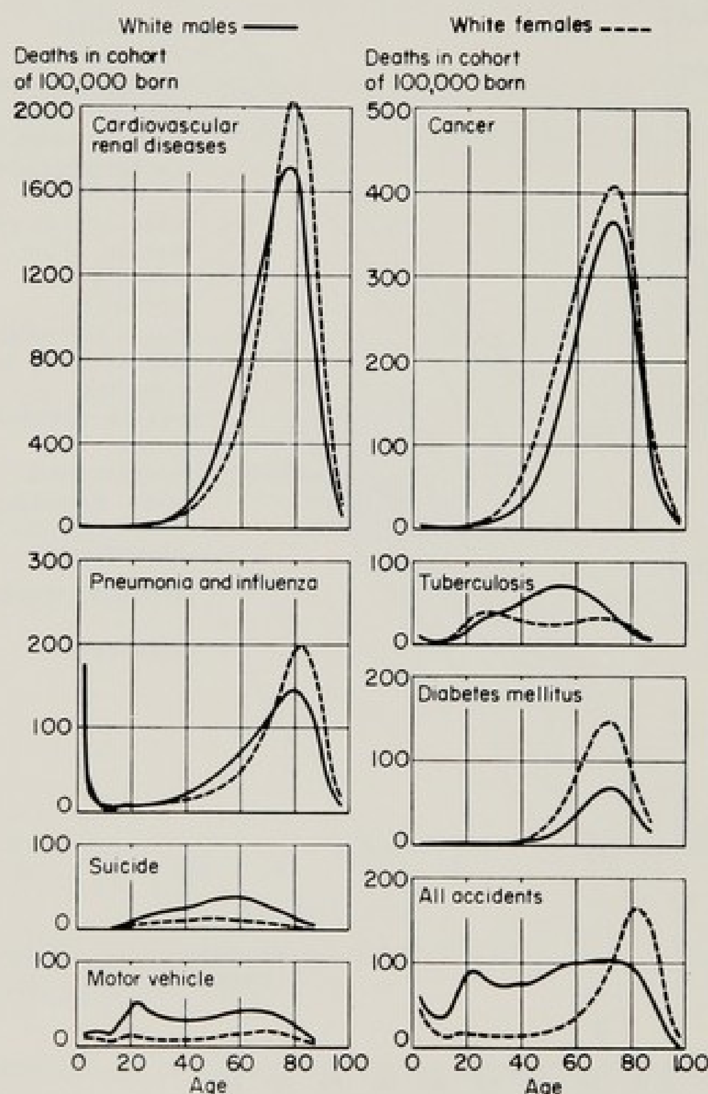

FIG. 3. Life-table deaths from the principal causes, United States, 1939–1941 (Dublin *et al.*, 1949).

quency distributions of deaths due to different processes are examined (Fig. 3). The mode value for populations which die only from respiratory infections and accidents (females) is approximately 81 years. Thus, 81 years may be taken as a value for the life-span of man.

Aging of a human population is programmed to the extent that the death rate doubles at regular intervals and a large number of pathologic

processes evolve and occur on schedule. Although the processes causing debility and death in older populations appear to be quite different from one another, they progress and cause death at approximately the same rates and during the same late periods in life. In addition, some of these processes represent the development of specific lesions while others apparently result from a decline in resistance factors. Deaths resulting from pneumonia are an example of the latter. The observation that different types of lesions and declining resistance characterize an aging population, suggests that one or a small number of underlying basic aging changes are occurring and that causes of death are complications of the basic changes.

Considerable attention has been directed toward age-related changes in physiological functions and processes responsible for homeostasis in human populations. Many careful studies have been performed on functional capacities in populations of varying ages. These studies have been reviewed previously (Shock, 1960a,b; Hobson, 1957; Kohn, 1963) and will not be described in detail here. The pattern which emerges is one of progressive decline in all functions from the optimal level attained in young adulthood. Respiratory, cardiovascular and excretory systems show a marked decline in efficiency with age. The aging human being becomes progressively less effective in regulating body temperature, blood pH, blood glucose levels, gland secretions, and reactivity of special senses. Maintenance of homeostasis becomes increasingly precarious, particularly when equilibria are upset by metabolic or environmental stresses.

We have now characterized an aging population as one with a sharply defined life-span, a population which dies off at a rate that doubles at regular intervals, develops a number of specific pathological lesions, and demonstrates a progressive decline in resistance factors and in efficiency of essentially all physiological mechanisms. Any changes at the cell or tissue level proposed as causes of aging must explain these characteristics of an aging population.

Role of Cells in Animal Aging

The characteristics of aging populations just described are by no means very subtle. An aging animal changes so drastically that just by observation we can recognize this change and can frequently guess its age quite closely. We would therefore expect an early agreement on the part of investigators as to which systems, cells, or tissues undergo the important underlying alterations. Such agreement has not been reached although enough data is now available to enable us to discard some untenable views at least until some unanticipated supporting evidence appears.

When an age-related alteration is discovered at the cellular or tissue level, the question arises whether or not it has an important causal role in the various debilities of aging. In most cases it is practically impossible to devise experiments which could prove or disprove such a causal relationship, and use must be made of indirect methods. Is the basic alteration a true aging process? Does it demonstrate universality within a population, and does it progress? Reference can be made to postulates analogous to those of Koch relating microorganisms to disease: Is the basic change always present when the debility is present and does it always precede the debility? Is the debility always present when the basic change is present, and is there a positive correlation between extents of the two? When two or more basic changes are considered as causes of a given debility, the one which best satisfies such postulates should be favored.

A theory which does not appear to age itself states that a higher animal ages and dies because its cells age and die; aging is the sum total of cellular aging and when we know why a cell ages we will know why a human being ages. The very remarkable achievements in studies of the DNA code and the sequence of events resulting in protein synthesis have helped to popularize this theory. Since these intracellular mechanisms can provide answers to so many important questions in biology, it has been assumed by many that their study will result in a solution to "the aging problem." Aging is frequently discussed in terms of somatic mutations, altered instructions, defects in information readout, etc. Although this type of approach is of value in considerations of growth cessation and of cellular aging, it will be argued that the aging of higher animals is not caused by aging of their cells, and that other more plausible explanations are available.

The role of cellular aging should be considered against a background of two concepts. First, aging of a higher animal is a generalized process occurring throughout the body. This is apparent from data on diseases and physiological mechanisms discussed above. No available information enables us to ascribe age-related debilities to a single organ, and individuals seldom, if ever, die from anything that could be considered uncomplicated aging of a given organ. Second, the reserve of cells in various organs is enormous. It is well known that a human being can survive without symptoms with less than 40% of his liver, part of one kidney, one lung, fractions of stomach and intestine, etc. Thus, if aging of the intact animal results from aging of its cells, we would expect to find cell death or loss of cellular function in organs throughout the aging

body, and we would expect the loss of very large numbers of cells—perhaps 50% or more of those present in the young adult.

Even though an animal is aging, many of its cells actually appear to be young. These are the cell populations which are constantly being replaced. Epithelial surfaces such as those of the skin and gastrointestinal tract consist of relatively young cells which continually replace degenerate cells. The same is true of both erythroid and myeloid elements of the blood. It has been estimated, for example, that the epithelium of the human gastrointestinal tract is replaced every 3 to 6 days (Lipkin, 1965). There is no evidence that such cell turnover is significantly hindered with increasing age (Grant and Le Grande, 1964), or that the newly formed cells in an older individual are themselves aged. Other cell populations which have a slower or less clear-cut turnover rate, demonstrate a striking capacity to react to injury or cell loss by accelerated cellular proliferation resulting in regeneration and restored organ function. The liver has been particularly well studied in this regard, and its ability to reconstitute quickly the original mass after removal of 70% has been frequently documented. This regenerative capacity is not significantly diminished in old age (Bucher and Glinos, 1950). Loss of cells or cellular functions with age in any organ or tissue in which there are cells capable of dividing should not result in any long term changes in cell number or in organ or tissue function. Cells which might be expected to show some significant changes related to the age of the animal are those which normally do not divide in the adult. Some skeletal muscle proteins, however, have a high rate of turnover and it has been shown that some proteins of neurons have turnover rates comparable to those of plasma proteins (Davison, 1961). Organelles have also been shown to turn over faster than the cells which contain them (Fletcher and Sanadi, 1961) indicating that many components of old cells are not necessarily old themselves. Other components, however, such as proteolipids of the central nervous system (Davison, 1961) are known to have essentially no turnover in fixed post mitotic cells. If cell aging occurs to a degree sufficient to cause debilities in the intact animal it should occur in these cells and as a result of changes in their nondynamic constituents.

Many studies of age-related changes in cells of the body have been carried out, including fixed post mitotic cells, and consideration of the findings should help us determine the role of cell aging in the aging of a higher animal. These findings have been reviewed by several authors (Bourne, 1957; Strehler, 1962; Kohn, 1963) and will not be described here.

Cells at scattered sites in aging animals have been reported to show a variety of ultrastructural alterations, accumulation of pigmented substances, and slight changes in composition and enzyme content. It would perhaps be more useful to emphasize what has not been described in aging tissues. No overall loss of cells has been noted which could begin to depreciate the known reserve in specific vital organs. Similarly, neither losses of enzymes nor of metabolic activity severe enough to depreciate the reserve or to result in functional debility of an organ, or to demonstrate the occurrence of numerous changes such as somatic mutations have been described. These conclusions on the triviality of cell changes in aging of the animal are based on a comparison between the generalized deterioration of an aging mammal in terms of physiological mechanisms, diseases and death rates, and the extent of cellular changes which has actually been found. When the postulates mentioned above are considered, relating basic change to debility, the relationship between cell and animal aging becomes even more tenuous.

The notion that cell aging causes animal aging has been kept viable largely by findings in two closely related areas which cannot be ignored in a discussion of aging mechanisms. First, if mammals are given low doses of ionizing radiation early in life their lifespans are shortened in a manner which simulates accelerated aging (Upton *et al.*, 1963; Jones and Kimeldorf, 1964). The second line of evidence comes from the work of Curtis (1963) who by elegant procedures demonstrated an increasing number of chromosomal aberrations in liver cells with increasing age of mice. Relating his findings to the radiation data, Curtis advanced the thesis that aging in general could be best explained on the basis of somatic mutations. Mice, however, do not die of or with liver failure, and of all sites in the body, the liver is probably the one with the least age-related change in terms of function or disease, presumably because of the cellular reserve and regenerative capacity mentioned above. Thus, chromosomal aberrations in this organ cannot be used to explain the aging of a mouse. Curtis (1963) also found that irradiated mice very quickly demonstrated large numbers of chromosomal aberrations in the liver, but were not rapidly aged by any criteria; in time, their tissues showed a reduction in the number of altered cells. These results suggested to Curtis that, while the liver was useful in demonstrating the cellular alterations, the important mutations causing aging were in organs with different rates of cell division. Curtis, however, also presented data showing that mice receiving near-lethal doses of the mutagen, nitrogen mustard, for two-thirds of their life-span, did not have shortened life-spans and did not

have altered liver cells although mutations occurred in several other sites. This led Curtis to the conclusion that the brain, which was not studied, was a possible site of the important aging mutations. There is no evidence available, however, that relates the debilities of aging to brain failure. Rather than trying to keep alive the somatic mutation theory of aging by making numerous assumptions, we should perhaps account for the observations by making the single assumption that, while aberrations occur in cells with time, they are probably of no great importance in the aging of the intact animal.

In regard to life shortening induced by ionizing radation, the patterns of disease in irradiated mice differ in several ways from those in a naturally aging population (Alexander and Connell, 1963). These authors point out that in acceleration of normal aging the latent period of all diseases should be advanced and their incidence should be unaffected. In addition to the possibility that radiation appears to accelerate only certain aspects of aging, there is a strong likelihood that radiation does not affect aging by causing cellular changes of the type usually considered to be mutations. Casarett (1963) has described radiation effects on small blood vessels which result in a widespread arteriolocapillary fibrosis. From the discussion to follow, there are reasons to believe that interference in vascular function by connective tissue is an important cause of animal aging. Since both ionizing radiation and natural aging are associated with generalized changes in connective tissue, it would not be surprising to find similarities in the consequences of the two.

Role of Connective Tissue in Animal Aging

In comparison with changes of equivocal significance found in cells as a function of the age of the animal, very striking alterations have been described in connective tissue fibrous elements. These changes appear to be generalized, and when considered in relation to the distribution, metabolism, and function of connective tissue proteins, they appear sufficient to explain most of the major manifestations of mammalian aging. A great deal has been learned about collagen largely as a result of early work by chemists in the leather, glue, and gelatin industries who determined the composition and properties of this substance, while biologists devised methods for straining out or otherwise eliminating connective tissue so that they could study cellular processes. Less is known about elastin, but in regard to age changes those so far described for elastin appear analogous to the changes in collagen which will be described in some detail below. The most conspicuous alterations in aging elastin are an increased

mineralization, increased cross linking, and an increased accumulation of a fluorescent material which may participate in the cross links (Partridge *et al.*, 1963; LaBella and Lindsay, 1963; Yu and Blumenthal, 1963; Eisenstein *et al.*, 1964; Miller *et al.*, 1964). Studies of collagen have progressed beyond those of elastin, and emphasis will be placed on collagen in this discussion. Alterations in collagen are undoubtedly of importance in their own right, and age-altered collagen might also serve as a model for aging of the closely related reticulin and other stable fibrous proteins including elastin.

Collagen constitutes about one-third of the total body protein. Histological sections stained by aniline blue show collagen distributed around cells, within and around blood vessel walls throughout the body, and as the major component of bone, cartilage, tendon, and skin. In organs it can be viewed as part of a matrix in which the cells are imbedded. The intimate association between collagen fibrils and small blood vessels can be appreciated in electron micrographs (Fig. 4). Collagen is an unusual protein in that glycine contributes about one-third of the amino acid residues and proline and hydroxyproline another third. Tropocollagen, the collagen molecule, has a molecular weight slightly over 300,000 and is released from cells into the extracellular space as a rod with a length of 2800 Å and a diameter of 14 Å. Extracellularly, tropocollagen molecules undergo an end-to-end polymerization and lateral aggregation to form fibrils and fibers. The characteristic 640 Å periodicity of fibrils seen in the electron microscope is caused by adjacent molecules being out of phase with each other by one-forth the length of a molecule (Schmitt, 1959). The collagen molecule is composed of three polypeptide chains of about 100,000 molecular weight each in a helical arrangement. In some collagens two of the chains appear identical (Piez *et al.*, 1961), while at least one type of collagen has been reported to contain three chains all differing in amino acid composition (Piez, 1964).

When extracellular collagen matures to form fibrils and fibers there is an increase in covalent cross links between polypeptide chains within tropocollagen and the formation of intermolecular cross links between tropocollagen molecules (Piez *et al.*, 1961). There is some evidence that both intra- and intermolecular cross links form by single continuous processes (Bornstein *et al.*, 1964). As collagen matures, it becomes increasingly insoluble. A high proportion of newly synthesized collagen is extractable by neutral salt solutions and dilute acids while only traces of fully mature collagen are extracted by these agents. A similar maturation process in which fibrils form from soluble collagen subunits and become

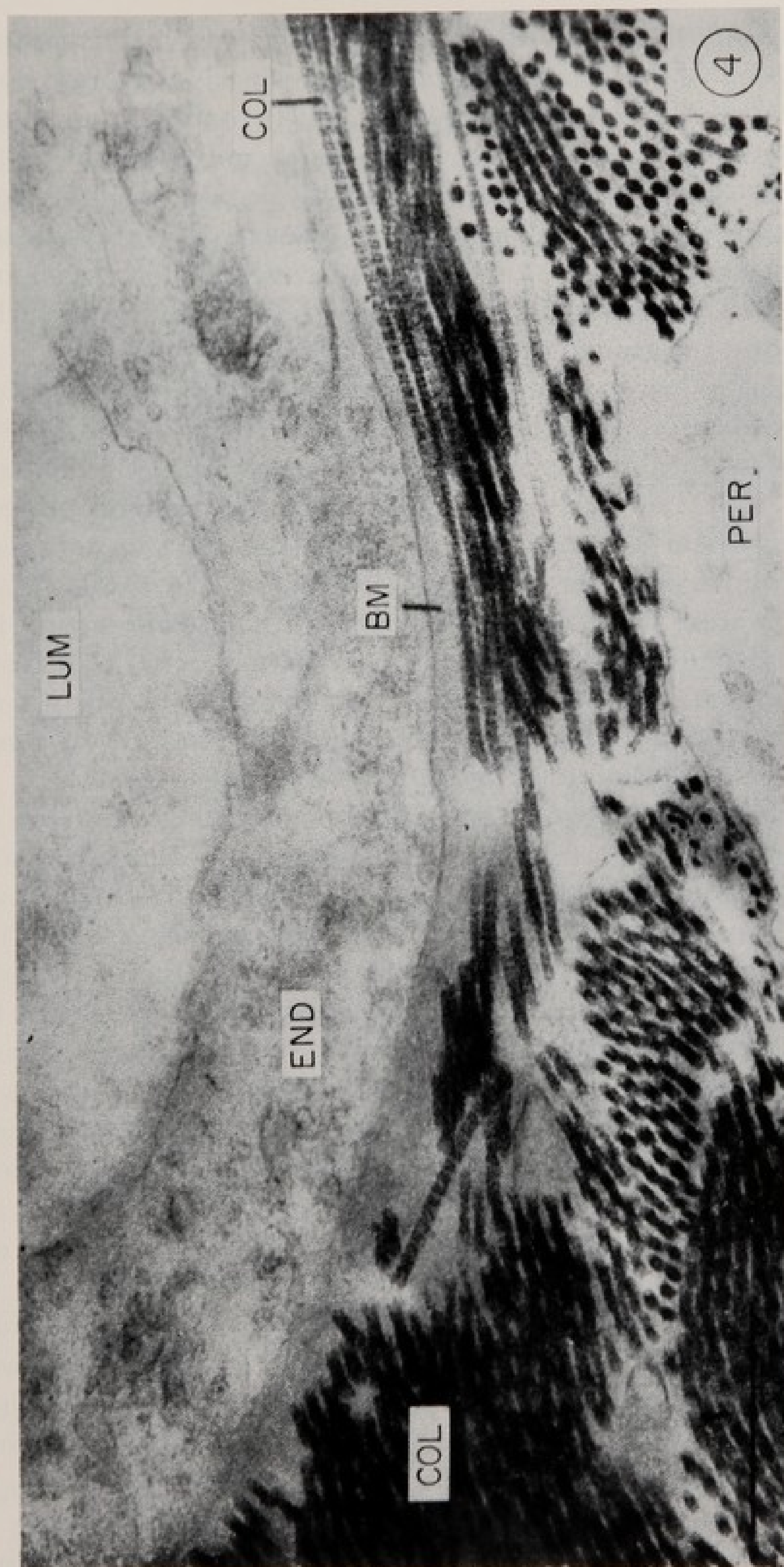

FIG. 4. Portion of capillary of hamster cheek pouch. The basement membrane (BM) is seen as a homogeneous band limited by collagen fibrils (COL); LUM, lumen; END, endothelium; PER, perivascular cell (Fernando *et al.*, 1964).

increasingly insoluble when incubated at 37°C can be demonstrated *in vitro* (Gross, 1958b). When an animal is growing it has tissue pools of newly synthesized soluble collagen. When growth stops, either naturally, or because of dietary restriction, new collagen is not added to the pools and the soluble collagen disappears, presumably because it is incorporated into the mature insoluble collagen (Gross, 1958a). The relationship of growth to aging of collagen can thus be appreciated. So long as an animal is growing it will contain some young collagen, the amount of which will be dependent on the growth rate.

Mature collagen has an extremely low turnover rate at most sites under the usual physiological conditions. It is quite likely that most of the body collagen does not turn over at all (Neuberger and Slack, 1953; Thompson and Ballou, 1956). Collagen is not degraded by the common proteolytic enzymes of tissues at physiological pH levels, and collagen morphology and composition have been found well maintained in fossils (Wyckoff *et al.*, 1964). Mature fibers are easily deformed by laterally acting forces but cannot be significantly extended; they have a very high Young's modulus (Kohn and Rollerson, 1959b; Harkness, 1961). Such a metabolically inert and insoluble material would be expected to undergo change with the passage of long periods of time.

The most conspicuous age changes in collagen which occur at quite precise times and rates are in physical properties. Although scattered references were previously made to age-related changes in collagen much recent work was stimulated by the observations of Verzár (1957) and Banfield (1956). Varzár made use of the phenomenon of thermal shrinkage of collagen fibers. He found that with increasing age of rat tendon fibers the amount of weight required to inhibit such shrinkage increased markedly. Banfield observed that with increasing age human tendon fragments lost the ability to swell in dilute acid. Both of these observations can be explained on the basis of an increased cross linking between collagen molecules.

We have found the swelling properties of collagen to be useful manifestations of subunit organization and have extended Banfield's work through studies of age-related variation in such properties in human collagen. When collagen is placed in a hydrochloric acid solution at pH 2.5 it imbibes water and swells to a marked degree. This separation of subunits is caused by the establishmnt of a Donnan equilibrium characterized by an excess of diffusible ions inside the tissue which, by their tendency to diffuse out, exert pressure on components of the tissue. The extent of swelling provides a measure of the modulus of elasticity (Proctor

and Wilson, 1916) and indicates the degree to which factors are present which hold subunits together. When swelling capacity was studied as a function of the age of human collagen (Kohn and Rollerson, 1958), it was found to remain at a high level until approximately 30 years of age when it decreased quite rapidly until 50 years of age and thereafter declined at a slower rate (Fig. 5). By comparison, Young's modulus, determined by

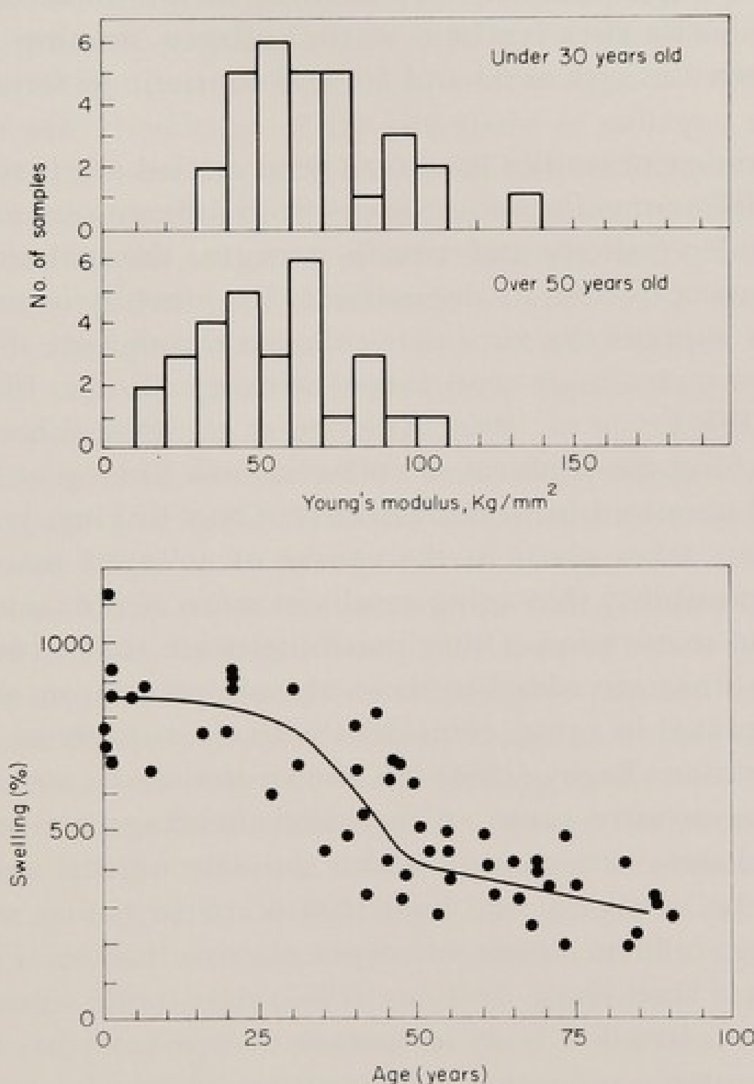

FIG. 5. Top: Young's modulus of human tendon fibers as a function of age. Bottom: Osmotic swelling ability of human tendon at pH 2.5 as a function of age, expressed as percentage increase in weight (Kohn and Rollerson, 1958, 1959b).

measuring the increase in length of collagen fibers when weighted at one end, was found to undergo a slight decrease with age which was hardly of statistical significance (Fig. 5). These changes were consistent with the view that cross linking occurred between adjacent collagen molecules with age. It was of particular interest that the changes began at 25–30 years

of age, e.g., after growth had ceased and after all of the collagen had matured. In subsequent studies it was found that swelling caused by thermal denaturation varied in both rate and extent as functions of age. By correlating thermal and Donnan equilibrium swelling data, it could be shown that collagen passes through four stages of increasing rigidity during the human lifespan (Kohn and Rollerson, 1959a). This increasing cohesion of collagen subunits was ascribed to intermolecular cross links which form shortly after synthesis of the collagen, develop to the greatest extent between the ages of 30 and 50, and continue to form after the age of 50.

A large number of studies have now been carried out involving changes with age in different collagenous tissues from different animals. Properties studied include elasticity and tensile strength, thermal shrinkage, solubility, and susceptibility to degradation by proteolytic enzymes. Accumulated data support the view that collagen throughout the mammalian body becomes increasingly cross linked with age (Kohn, 1959; Kohn and Rollerson, 1960; Fry *et al.,* 1964; Kulonen *et al.,* 1963; Schaub, 1963).

In approaching the problem of collagen cross linking at the molecular level, several questions arise immediately. Cross linking, both intra- and intermolecular, takes place in the course of collagen maturation. This suggests the possibility that aging results in more of the same type of links which form in maturation. Other possibilities are that with age different types of cross links are added to those already present, or that new types of links are formed in aging, coincident with the rupture of bonds formed during maturation. Rigby (1964) has shown that cyclic stresses applied to collagen fibers *in vitro* cause an increased shrinkage temperature and a more oriented structure, changes which simulate natural aging. This suggests that cyclic stresses of the kind that occur in tissues may over long periods of time influence rates and types of cross linking. The interesting observation has been made by Sinex (1957) that stable substances such as collagen are incubated at body temperature for many years and would be expected to undergo some thermal denaturation which might contribute to age changes. This possibility has been tested by experiments based on the assumption that if thermal denaturation at body temperature was a significant cause of collagen aging it should be possible to incubate collagen at elevated temperatures, i.e. 56°C, and reproduce all of the age-related changes *in vitro*. It turned out that elevated temperature did in fact cause agelike changes in swelling capacity and solubility. Thermodynamic calculations based on certain long range extrapolations suggested that thermal denaturation might explain the *in vivo* changes with age

(Kohn and Rollerson, 1959a,b). One of the striking age-related changes in collagen, however, a decreased susceptibility to the action of collagenase, was not reproduced by thermal denaturation (Kohn and Rollerson, 1960). Although it is difficult to conceive of thermal denaturation not occurring in a protein incubated at 37°C for 50 years, and some denaturation may take place in collagen, collagen aging cannot be explained on the basis of denaturation alone. The possibility exists that heat plays a partial role in causing the rupture of intramolecular bonds, for example, and freeing reactive groups which then participate in intermolecular cross links.

The bulk of our knowledge of the chemistry of collagen cross linking has come from studies dealing with the chemistry of collagen per se rather than from studies motivated by the desire to learn something about aging. This has resulted in the majority of investigations being carried out on collagen molecules and molecular subunits in solution. A large variety of different kinds of bonds at different sites has been proposed as a result of observations of such systems. These include bonds formed by a noncollagenous pepsin-sensitive peptide (Rubin *et al.,* 1963), ester links (Joseph and Bose, 1962; Bello, 1960), and aldehyde-mediated links in tyrosine-rich regions (de la Burde *et al.,* 1963; Schlueter and Veis, 1964). Although links of these types appear to form in maturation, the extent to which they form in the transition of mature collagen to old collagen is unknown. Old human collagen has been treated with pepsin in an attempt to rupture the proposed noncollagenous peptide links. In addition, old collagen has been treated with hydroxylamine to rupture ester or other hydroxylamine-sensitive bonds. According to the results of swelling experiments, neither of these treatments has caused "rejuvenation" of the collagen (Kohn, 1962). When a soluble fraction is obtained from collagenous tissue by extraction with neutral salt solutions or acidic buffers it contains fractions of young collagen regardless of the age of the individual from which the sample was obtained. Conclusions regarding aging of collagen have been drawn from studies of soluble collagen which represented less than 1% of total collagen in the tissue (Bakerman, 1964). In a recent study of total collagen, it was reported that a fluorescent substance and a yellow pigment increase with age while the tyrosine content decreases. This suggested that tyrosine residues become oxidized to a quinoid which participates in cross linking (LaBella and Paul, 1965). Old collagen is one of the most insoluble of biological substances. Very harsh treatment is required to solubilize it for study and there is a possibility that such treatment will abolish or alter the age-related changes. Nondestructive analytical methods such as wide-line nuclear magnetic

resonance and electron spin resonance spectroscopy and deuterium or tritium-hydrogen exchange may prove of value in describing the important intermolecular cross links which form as insoluble mature collagen is transformed into insoluble old collagen.

If it is assumed that intermolecular cross links form in collagen with age and thereby cause collagenous tissue to become more rigid throughout the body, it becomes necessary to relate such alteration to the debilities of aging, e.g., the decreased efficiency of homeostatic mechanisms, decline in resistance, and increased incidence of certain diseases. The establishment of such a relationship depends on knowledge of the physiological role of collagen. Because of its physical properties and distribution, we would assume that collagen is of importance in providing strength and maintaining the structure of organs. It is also apparent from the distribution that blood vessels pulsate and muscle cells contract within a framework of collagen, and that any substance passing between cells and blood vessels must move through collagenous extracellular material. Several studies have indicated that collagen fibers are oriented so that they bear stresses and provide structural limits to deformation, particularly in such mobile organs as the lungs and blood vessels (Harkness, 1961; Mead, 1961; Wolinsky and Glagov, 1964). Growing animals given a lathyrogenic agent such as β-aminopropionitrile develop collagen which does not mature properly because of inadequate cross linking (Martin *et al.*, 1963). Such animals with defective collagen are unable to maintain elevated blood pressure when injected with norepinephrine (Kohn and Rivera-Velez, 1965). This suggests that mature collagen plays a role in stabilizing or potentiating reactions of small blood vessels although the changes caused by lathyrogenic agents are not understood well enough to exclude other possible mechanisms of action.

The relating of collagen cross linking to debilities of age depends on the argument that such cross linking causes a greater rigidity of tissues and results in defective diffusion of substances between cells and vascular spaces. Defective diffusion could result from loss of tissue elasticity and movement within tissues so that substances are slowed in their passage, or could result from barriers provided by the more densely cross linked collagen per se. That age-altered collagen causes the organ or tissue which contains it to become more rigid is suggested by the observation that human myocardium loses osmotic swelling ability with age (Kohn and Rollerson, 1959c) and that the age-related difference is abolished by treatment of the tissue with bacterial collagenase (Kohn and Rollerson, 1959d). The collagenase used in these studies may have contained other

proteinases and more work of this type should be performed with a highly purified enzyme. From this and from the studies of age-related changes in collagen described above, such as a decrease in Donnan equilibrium swelling, it would appear that both collagen itself and tissues which contain it do become more rigid with age. Because of the distribution and suggested physiological role of collagen, an age-related increase in rigidity would be expected to cause alterations in diffusion. Some evidence is accumulating which demonstrates altered tissue permeability with age although the role of collagen has not been defined. Thus, diffusion of a silver proteinate into cartilage, a tissue rich in collagen, decreases with age (Stockwell and Barnett, 1964). When a labeled protein is injected intravenously, the amount found within perfused myocardium decreases with age, suggesting the presence of a barrier to diffusion (Sobel *et al.*, 1964). In the absence of methods by which proof of the causal role of collagen in altered diffusion can be obtained, it is necessary to acquire an overwhelming amount of circumstantial evidence. A useful experiment might involve *in vivo* determination of age changes in metabolic or physiological activity in an intact organ in which blood vessel, connective tissue, and cell relations are intact, followed by a study of biochemical processes in homogenates in which the metabolic systems are freed from their connective tissue environments. If significant age changes in intact organs were not observed in homogenates, support would be obtained for the view that the extracellular environment or cell membranes were causes of aging. In a sense these types of experiments have already been performed. From the discussion earlier, it is clear that the functional activity of organs such as kidney declines markedly with age, but studies of kidney metabolism by biochemical techniques have not revealed any very striking age-related changes.

A conclusion consistent with available data is that in addition to increased rigidity of collagenous tissue with age, increased cross linking of collagen causes altered diffusion in tissues. The probable relationships of these changes to debilities of aging have been described previously (Kohn, 1963) and will only be summarized here. It has been argued that connective tissue alterations are sufficient to explain almost all of the characteristics of aging human beings. The age incidences of hypertension and arteriosclerosis could be explained, respectively, on the basis of increasing rigidity of small vessels and on decreasing diffusion of nutrients and other materials within vessel walls with consequent inflammation and complications caused by the trapping of minerals and lipids. Decreased resistance to infection and trauma might be due to the inability of hormones, nu-

trients, and antibodies to reach critical sites in the appropriate concentrations and inefficient removal and detoxification of harmful substances. Similarly, the decline in efficiency of physiological and homeostatic mechanisms would be due to the lowered reactivity of small vessels because of their more rigid connective tissue matrix and to the sluggish passage of metabolites, hormones, and chemical messengers from cell to cell or cell to vessel through connective tissue.

A significant age-related change which, at the present time, is difficult to explain on the basis of connective tissue alterations, is the increased incidence of malignant neoplasms. It has been pointed out, however, that malignancies lack the universality of a true aging process within a population, do not follow a Gompertzian type curve in regard to cause of death, and do not play a very important role in determining the mean life-span of a human population (Kohn, 1963).

A more serious problem encountered by the view that aging connective tissue causes aging in animals is the difference in life-span between different species of mammals. The mean life-span of the rat may be $2\frac{1}{2}$ years and that for man 70 years. When the rat dies, its oldest collagen is the same age as infantile human collagen. Aging of collagen has been described as an extracellular phenomenon analagous in some ways to crystallization. Although cross linking of rat collagen with age has frequently been described, apparently no one has attempted to determine whether collagen from a given site of a $2\frac{1}{2}$-year-old rat is cross linked to the same extent as a similar sample from a 70-year-old man. It is not clear why the rat collagen should be any more cross linked than very young human collagen. A possible explanation is based on the knowledge that collagen molecules and fibers are laid down parallel to lines of stress in tissues, that cyclic stresses cause a more oriented structure of fibrils probably resulting in more cross linkages (Rigby, 1964), and that the life-span of mammals has a clear-cut inverse relationship to metabolic rates of different species (Sacher, 1959). The rat, having a higher metabolic rate than man, would have a greater rate of vessel pulsation and, in general, greater rates of tissue movement and a higher frequency of tissue stresses. This could result in a more rapid alignment of molecules and fibrils and more rapid formation of collagen cross links to the extent that rat collagen at $2\frac{1}{2}$ years is really comparable to human collagen 70 years old. Some support for this hypothesis has been gained by the recent observation that aging of collagen is slowed in hypophysectomized rats (Olsen and Everitt, 1965). A decreased metabolic rate would be one of the prominent changes in such animals.

We have some methods for testing the collagen theory of aging, as well as possibly lengthening the life-span. It should be possible to keep animals on a diet deficient in ascorbic acid so that maturation of collagen is slowed, but not deficient enough to produce symptoms of scurvy. Such animals should have a lengthened life-span. Also, the lathyrogen β-aminopropionitrile mentioned above, can be given to animals from the time of weaning in doses sufficient to slow down collagen cross linking but not enough to cause overt lathyrism. We have experiments utilizing the nitrile in progress. Groups of rats and mice are receiving β-aminopropionitrile fumarate in their drinking water from the time of weaning and their life-spans and mechanisms of death will be noted. The groups of animals are small since they are kept pathogen-free in isolators. Preliminary studies have been required to determine the dosage which does not cause lathyrism over long periods. The first group of rats, decimated by an air conditioning failure, now consists of four control and four experimental animals which were started on the nitrile in August, 1963.

Aging of Cells within Animals

Although the role of cell aging in the aging of a higher animal has been deprecated, aging of cell populations is a widespread biological phenomenon which satisfies the criteria of universality, progression, increasing age-specific death rates, and growth-relatedness. Very little work has been performed in an attempt to understand the mechanisms by which cells degenerate and die on schedule after they have stopped dividing and have attained a certain high level of specialization. In our present state of ignorance we can do little more than describe the phenomena of cell aging and consider some preliminary experimental data which might be useful in directing future investigations.

There are enormous differences between life-spans of different cell-populations within a higher animal. In man, neurons are capable of living as long as the individual while the granular leukocyte life-span is measured in days or hours. In terms of differentiation, differences such as these in cells which have both come from the zygote and presumably have the same genetic makeup, probably represent a maximum divergence in controls of gene expression. Cells which die in a higher animal appear to belong to at least two different types of populations. In one, cell life-span is quite distinct and degeneration appears programmed. In the second, the life-span cannot be defined and cell death appears to be a random-hit process with a probability of occurrence not directly related to cell age.

Cell death in the latter case appears to be accidental and is presumably due to breakdowns and failures of the machinery of cells which do not synthesize new DNA and cannot repair themselves. Death of neurons in the central nervous system appears to be such a random process. In the human being, neuron death is a steady process which is not accelerated with increasing age (Wright and Spink, 1959). In accord with this view of randomness is the observation that neurons of mice do not show a very significant dropping out over the life-span (Wright and Spink, 1959), probably because there is not sufficient time for a large number of intracellular accidents to occur.

The cell populations which age and die on schedule have been mentioned earlier in this discussion. Such populations may constitute almost the entire animal as in the case of hydra (Burnett, 1961), or exist at many sites in higher animals as exemplified by epithelial tissues and leukocytes in mammals. Characteristically, in these populations there is a focus of stem cells which are periodically or continuously dividing, giving rise to cells which no longer divide but which undergo morphological and chemical specialization. After passing through one or more well-defined and predictable phases of specialization, the cells degenerate and die. This sequence is not necessarily irreversible. At every step in the sequence, questions arise which are pertinent to an understanding of growth, differentiation, and aging processes. We have answers to none of these questions. No more is known of factors which cause cells to stop dividing than of factors responsible for division in the first place, although extracellular factors such as functional demand and negative feedback mechanisms appear to exercise control of cell division in some systems.

Even if a cell is not dividing there is no theoretical reason why it should not remain viable indefinitely. Intracellular accidents may be invoked but it then becomes necessary to explain how such accidents kill all mature granulocytes and cells of the gastrointestinal tract in a matter of days, but not all neurons in 80 years. Many other hypothetical mechanisms can be advanced: toxic metabolites accumulate, degradative enzymes are synthesized, or essential molecules are used up and not resynthesized. An old idea which has never been tested, is that in highly specialized cells so much of the cell's machinery is engaged in carrying out the special functions that there is not enough energy production left over for the maintenance of factors necessary for life, such as semipermeability of membranes or synthesis of enzymes. Finally, when a cell is no longer viable, the processes by which it degenerates are poorly understood. These are the processes

responsible for the degradation of cellular constituents and are presumably the same processes which occur in various types of cell injury and atrophy, and in normal turnover of organelles and proteins. From studies of protein turnover in atrophying cells, it would appear that such degradative processes are at least as important as synthesizing reactions in determining the size of some cells and the amount of cellular constituents present at a given time (Slack, 1954; Simon *et al.*, 1962).

Many measurements have been made of cell life-span, and various stages of differentiation and specialization occurring after the last division have been described for a variety of cell types. From the viewpoint of cell aging, however, it would appear that very few attempts have been made to discover why a cell degenerates and dies, or to discover the mechanisms by which its constituents are degraded. Our own work in this area was started with neutrophils obtained from the peritoneal cavity of the rat following injection of a saline solution. These cells do not divide, and constitute a population which degenerates on schedule and has a life-span of a few days. They were maintained under conditions believed to be very favorable and various metabolic and morphological characteristics were followed as a function of time *in vitro* (Kohn and Fitzgerald, 1964). The purpose was to define cell death in terms of specific cell function or property and to detect the earliest degenerative change. The latter might suggest an initial cause of degeneration and be a cause itself of subsequent alterations.

The cells degenerated both metabolically and morphologically at rapid rates as soon as they were obtained and placed in a tissue culture environment. Deterioration occurred earliest in lactate production and in succinate dehydrogenase activity, while alterations in cell permeability, morphology by light microscopy, and cell protein occurred at slower rates (Fig. 6). It was subsequently found by electron microscopy that all components of the cells underwent marked degeneration during 8 hours in culture. However, the cells could incorporate labeled amino acids into trichloracetic acid-insoluble material at a steady rate for 20 hours *in vitro* (Kohn, 1964b).

It thus appeared that death in these cells is not an all-or-none phenomenon, but has a different time course depending on the property under observation. Deterioration can be detected earliest in morphology by electron microscopy and in glycolysis and succinate dehydrogenase activity. It was of interest that although glycolysis and succinate oxidation depend on different enzymes, the ability to carry out both of these proc-

esses declines at approximately the same rapid initial rate. These results suggest either earlier defects in ability to synthesize various enzymes concerned with intermediary metabolism, or earlier accelerated rates for their degradation.

The very rapid degeneration of these cells *in vitro* suggested the possibility that they were not dying natural deaths but were being killed by the experimental procedures. Lactate production was used as a measure of viability, and the cells were treated in different ways including culture on siliconized and ordinary glassware, incubation in a complete culture medium, in serum, and in Ringers solution, and incubation in air versus

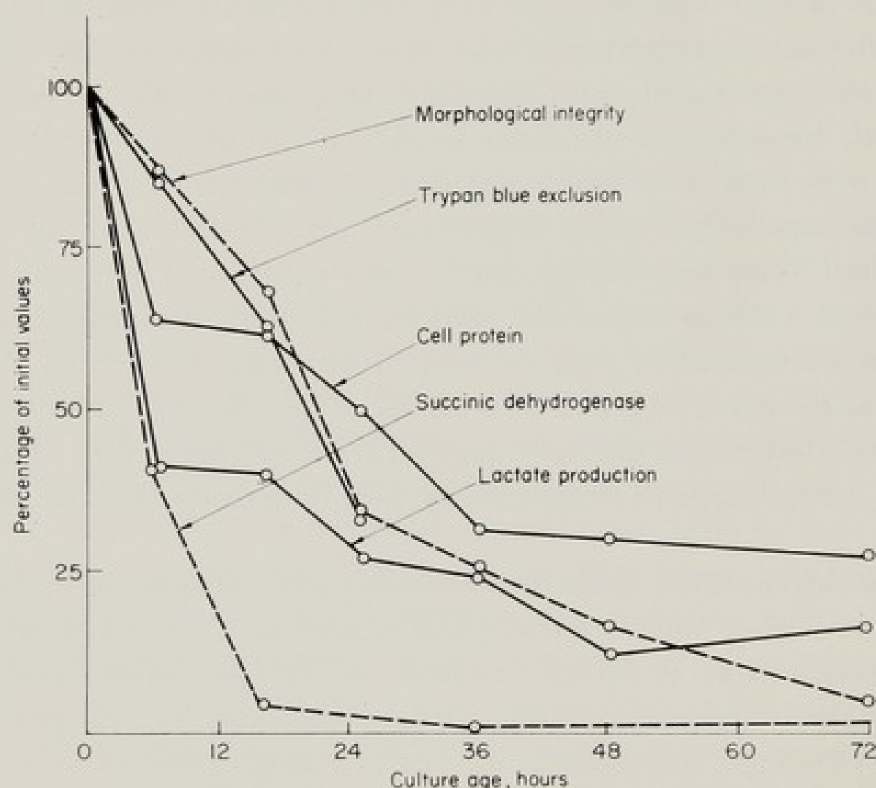

Fig. 6. Percentages of initial values for various leukocyte properties as functions of time *in vitro* (Kohn and Fitzgerald, 1964).

a mixture of nitrogen, oxygen, and carbon dioxide. None of these variables appeared to alter significantly the rate of decline in lactate production, suggesting that degeneration and death were processes intrinsic to the cells. Any additional role played by trauma or environmental change might be comparable to that played by similar factors *in vivo*.

Attempts were made to obtain information on mechanisms of cell degradation in dying neutrophils. Particular attention was given to the possible role of lysosomes because of the attractive hypothesis that such bags of hydrolytic enzymes are stimulated to release their contents which then degrade constituents of cells in cases of atrophy or cell injury (see

reviews edited by de Reuck and Cameron, 1963). Lysosomes appeared especially large and numerous under the electron microscope while neutrophils degenerated rapidly. Attempts to demonstrate increased intracellular protease activity by determinations of protein degradation products released into the medium during degeneration, however, yielded equivocal results. Increased autolysis could usually be detected but it varied in amount and time of onset from one experiment to another.

Another system was then chosen for the study of degradative processes. Skeletal muscle cells do not normally age and die, but when deprived of their innervation they provide a degenerating system which has several advantages over the leukocyte cultures: Large, uniform samples are obtainable, atrophy is very rapid and reproducible, and a great deal is known about muscle enzymes and structural proteins. The mechanism of muscle atrophy is also of interest because of the disease, progressive muscular dystrophy, which is characterized by atrophy and disappearance of cells in ways which simulate aging of a cell population. In addition, muscle contains the largest amount of mobilizable protein in the body. Mechanisms by which such protein is degraded in starvation, disuse and denervation atrophy, and muscular dystrophy are presumably identical or very closely related.

Previous studies suggested that muscle wasting in both denervation atrophy and dystrophy resulted from the accelerated breakdown of protein rather than inhibited synthesis (Slack, 1954; Simon *et al.*, 1962). In addition, an increase in lysosomal enzymes has been demonstrated in muscular dystrophy (Tappel *et al.*, 1962). Our work on denervated muscle was initiated by the supposition that we could use as substrate a well-studied major structural protein such as myosin which is known to disappear from muscle in atrophy and dystrophy, and isolate from control and atrophying muscle the lysosomal proteases which degrade it. This system could then be used to study factors which cause synthesis or activation of the degradative enzymes.

After section of the sciatic nerve in rats, lower leg muscles lose 50% of their protein in 12 days. Although 50% of the myosin is lost during this period, studies of extracted myosin revealed no intermediate stage in its degradation, and exhaustive attempts to identify a myosin-degrading enzyme in muscle were unsuccessful (Kohn, 1964a). We did obtain a partially purified protease from muscle granules which digested denatured hemoglobin but which appeared to have no activity toward pooled muscle proteins. This is consistent with a report by Bodwell and Pearson (1964) which states that muscle cathepsin does not digest the major structural

proteins of muscle. It was tentatively concluded at this point that loss of protein could not be explained on the basis of generalized proteolysis, that when muscle is removed from the animal important differences between atrophying and control muscle might be abolished, and that function per se might be an important factor in the regulation of protein loss. The latter view is supported by the observation of Cotlar *et al.* (1963) that stretching of a denervated muscle inhibits its wasting. A possible explanation of this is that in a flaccid muscle the highly polymerized proteins might be allowed to separate from one another and depolymerize spontaneously, or expose sites for enzymatic cleavage which would result in depolymerization to fragments which were still proteins but in a form which could leave the muscle. In muscle under tension, the polymers would be held in linear array, close enough to each other for short range forces to keep them in position. Such a mechanism would require no new enzyme system, but could utilize systems present in all muscle. This hypothesis was tested by incubating fiber bundles, both flaccid and under tension, and determining protein efflux into the medium. Tension was found to inhibit protein efflux markedly (Kohn, 1964a).

Although no degradation of myosin could be demonstrated in muscle, it was found that low levels of protein degradation occurred in homogenates of both control and atrophying muscle, and that autolysis was slightly but consistently greater in preparations from atrophying muscle. Recombination experiments with subcellular fractions indicated that at least three components were required for autolysis: one in the residue or myofibrillar fraction, one in the mitochondrial fraction, and one in the soluble fraction. The increased autolysis in atrophying muscle was dependent on all three components being obtained from atrophying muscle (Kohn, 1965). Electron microscopy has revealed no significant number of lysosomes in the mitochondrial fraction, but has demonstrated a difference between mitochondrial fractions from control and atrophying muscle in that the fraction from atrophying muscle contains fragments of myofibrillar protein. Recent experiments in which protein shifts have been followed during *in vitro* incubations of recombined fractions followed by refractionation, have indicated that in atrophying muscle there is an accelerated shift of residue or myofibrillar protein into the mitochondrial fraction, and that this shift requires the presence of both the mitochondrial and soluble fractions.

Our current working hypothesis, which seems consistent with all observations, is that the degradation of muscle structural proteins is initiated by the action of enzymes in the soluble and mitochondrial fractions which cause a depolymerization to fragments which are still large pro-

teins, and also cause the release of a small number of free amino acids or peptides. The fragments of myofibrillar proteins are further degraded, but not into units smaller than actin or myosin, after which they leave the muscle. The first depolymerization step is influenced by the physiological state of the muscle since fibrillar proteins in a flaccid muscle are more susceptible to attack by depolymerizing enzymes. Such an alteration in physiological state could be the major difference between atrophying and normal muscle. Zak and Drahota (1960), who followed the release of labeled methionine from muscle, similarly concluded that degradation was enhanced when the utilization of energy was limited and not dependent on greater amounts of proteolytic enzymes.

Our studies have not implicated lysosomes in degradation of muscle protein and there is some evidence that they do not participate in kidney necrosis (Nagel and Willig, 1964). Lysosome proteases are generally assayed according to their ability to degrade certain peptides and denatured hemoglobin, and apart from the demonstration that preparations from liver can digest some liver proteins (Sawant *et al.*, 1964), there is little reason to accept the view that lysosomes play an important role in the degradation of cell constituents throughout the body. Cathepsins appear in increased amounts in virtually every tissue undergoing degeneration, but whether this represents an increase in their synthesis or merely the fact that they are spared from degradation, and whether they play a trivial or important role in degradation is not known. There is no reason why mechanisms of degradation in different types of cells must be identical or even closely related, but it would seem quite uneconomical for a large number of different mechanisms to evolve to carry out similar processes in different cell populations.

In regard to cell aging, cells may degenerate and die because alterations in physiological state allow degradative processes to become dominant. Such altered states could arise because energy in specialized cells is used for specialized functions and there is not enough available for the maintenance of structural proteins in their proper state of alignment. Present knowledge enables us to consider these problems in only the most general terms, and probably the appropriate generality to be gained from the above discussion is that problems of cell aging constitute a neglected and challenging area in developmental biology.

Summary

An aging process can be defined as one which occurs in all members of a population under consideration, which has its onset or accelerates with

the slowing down of growth, and which progresses and is not reversible under usual physiological conditions. Progression distinguishes aging from most other biological processes and is responsible for the debilities and death of systems which age. Such processes occur from the molecular level to that of the intact higher animal and are seen most characteristically in systems which stop growing after attaining a high degree of complexity, and which contain metabolically inert components.

Aging in higher animals such as man is manifested by a clearly defined life-span, a logarithmic increase in rate and probability of dying with time, decreased efficiency in physiological processes, increased incidence of certain diseases, and increased mortality from other diseases. Aging of cells within a higher animal, from both a priori considerations and evaluation of experimental data, is not sufficient to explain these manifestations of aging. From data concerning the distribution, metabolism, and age-related changes in fibrous proteins of connective tissue, it is proposed that progressive cross linking of collagen, and possibly elastin, is the major cause of aging of higher animals, probably because of its effect on diffusion processes. Although considerable information has been gained about cross linking during maturation of collagen, it is necessary to describe the cross links which form as mature insoluble collagen is transformed into old collagen. The role of collagen can be tested and aging can possibly be inhibited by keeping animals on an ascorbic acid deficient diet or a diet containing a lathyrogenic agent.

Aging of cell populations also depends on growth cessation and should be distinguished from non-age-related deaths which occur in some non-dividing cells and which presumably result from intracellular accidents. Degeneration and death appear programmed in some cell populations and these age changes can be characterized in terms of enzyme activity and ultrastructural alterations. Such changes appear closely related to processes responsible for the degradation of cell constituents. By using denervated muscle as a model, evidence was obtained indicating that the degradation of structural proteins is initiated by alterations in physiological function which facilitate depolymerization of proteins by a complex enzyme system normally present. It is proposed that cell aging might be initiated by the inability of specialized cells to maintain highly polymerized structural units in their normal physiological state. The present state of ignorance regarding cell aging and the challenge this presents are emphasized.

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Author Index

Numbers in italics indicate the pages on which the complete references are listed.

A

Aach, H. G., 73, *91*
Aaronson, S., 141, 154, 181, *183*, *187*
Abbott, M. T., 20, *38*
Abrams, R., 28, *33*
Alberts, B. M., *34*
Albright, J. F., 247, *290*
Alexander, M., 22, *35*
Alexander, P., 303, *321*
Allan, R. K., 181, *187*
Allen, E. H., 22, *32*
Allende, J. E., 24, *32*, *36*
Ambrose, E. J., 277, *286*
Ames, B. N., 31, *32*
Anders, M., 20, 22, 28, *34*, *35*
Anfinsen, C. B., 58, *60*
Angevine, D. M., 243, *288*
Ansley, H. R., 113, 114, 123, *124*, *125*
Anthony, D. D., 22, *32*
Apgar, J., 28, *35*
App, A. A., 141, *183*
Arber, W., 28, 29, *32*, *34*
Arlett, C. F., 201, *210*
Arlinghaus, R., 24, *32*, *36*
Arnstein, H., *32*
Aronson, A. I., 71, *91*
Aronson, J. F., 223, *240*
Attardi, G., 30, *32*
Atwood, K. C., 25, *37*, *38*
Auerback, R., 246, 247, 280, *286*
Aurischio, A., 20, *38*
Axelrod, D., 103, *104*
Ayer, J. P., 304, *321*

B

Bakerman, S., 309, *321*
Ballieux, R. E., 41, *60*
Ballou, J. E., 306, *324*
Bancroft, J. B., 71, 85, *91*, *94*
Banfield, W. G., 306, *321*
Bang, F. B., 278, *287*
Barnett, C. H., 311, *324*
Barnett, L., 24, *33*, *34*
Baron, L. S., 177, *187*, *189*
Barondes, S. H., 23, *33*
Barski, G. S., 227, *238*
Basilio, C., 31, *38*
Bautz, E., 79, *92*
Bautz-Freese, E., 79, *92*
Bavetta, L. A., 22, *33*
Bawden, F. C., 77, 88, *91*
Beadle, G. W., 198, *210*
Beasley, J. O., 123, 124, *125*
Beaudreau, G., 88, *94*
Beisson-Schecroun, J., 209, *210*
Bekhor, I. J., 22, *33*
Bell, J., 66, *93*
Bello, J., 309, *321*
Benacerraf, B., 39, *60*, *63*
Bender, M. A., 114, 116, 117, *127*
Ben-Shaul, Y., 132, 136, 137, 138, 139, 141, 142, 143, *184*
Bensky, B., 154, 181, *183*, *187*
Benzer, S., 24, *33*, *38*
Berg, P., 20, 22, *33*, *37*
Berger, C. A., 110, *125*
Bernier, G. M., 41, *60*
Berquist, P. L., 27, *33*
Bertrand, H. A., 223, *238*
Best, R. J., 73, *91*
Bigelow, B., 40, *61*
Biggers, J. D., 294, *321*
Billeter, M. A., 88, *91*, *94*
Binaghi, R. A., 39, *63*
Bishop, C. B., 278, *289*
Bishop, J., 24, 31, *33*
Biswas, B. B., 28, *33*
Blakely, L. M., 227, *240*
Bloch, D. P., 114, *125*
Bloch, K., 141, *185*
Bloch, K. J., 39, *60*
Blumenthal, H. T., 304, *324*
Bockstahler, L. E., 84, 85, *91*
Bode, O., 66, *93*
Bodwell, C. E., 317, *321*
Bold, H. C., 144, *184*
Bolton, E. T., 24, *33*, 103, *104*
Bonner, J., 114, *125*
Boothroyd, E. R., 122, *129*
Borek, E., 38, *33*, *34*, *36*, *37*

Bornstein, P., 304, *321*
Borst, P., 88, *94*
Bose, S. M., 309, *322*
Bourne, G. H., 301, *321*
Boy, de la Tour, E., 136, *185*
Brakke, M. K., 66, *91*
Braun, A. C., 227, *238*
Braun, R., 177, *184*
Brawerman, G., 140, 141, 169, 173, 180, 181, *184, 185, 188*
Bremer, H., 31, *33*
Brenner, S., 21, 24, 27, 30, *32, 33, 38,* 84, *91,* 118, *126*
Brewbaker, J. L., 221, *238*
Bridges, C. B., 220, *238*
Briggs, R., 219, 221, 224, 225, *238, 239*
Bristow, J. M., 228, *238*
Britten, R. J., *36*
Brown, B. J., 31, *35*
Brown, D. D., 25, 27, *33, 35*
Brown, J. S., 142, *184*
Brown, R., 177, *184*
Bucher, N. C. R., 301, *321*
Buckley, C. E., III, 58, *60*
Burdon, R. H., 88, *91, 94*
Burger, R. M., 181, *189*
Burgi, E., 97, *105*
Burka, E. R., *36*
Burnett, A. L., 292, 314, *321*
Burnham, B. F., 180, *184*
Burns, V. W., 225, *238*
Burtin, P., 41, *60*
Busch, H., 115, *125*
Buttin, G., 100, *104*

C

Cahoon, M. E., 176, *187*
Caldwell, K. A., 317, *324*
Calef, E., 97, 99, *105*
Callan, H. G., 117, 118, *125*
Cameron, I. L., 121, *125*
Cameron, M. P., 317, *321*
Campbell, A., 97, 99, *105*
Carbonara, A. O., 40, *60*
Carell, E. F., 141, 180, *184, 188*
Casarett, G. W., 303, *321*
Caspar, D. L. D., 85, *91*
Catcheside, D. G., 209, *210*
Cavalieri, L., 109, 116, *125*
Cebra, J. J., 44, *61*
Cecere, M. A., 31, *38*
Chamberlin, M., 20, *33*
Chapeville, F., 24, *33*
Chargaff, E., 140, 173, 181, *184, 188*
Chase, S. S., 218, 236, *238*
Chatterjee, B. R., 293, *321*
Chayen, J., 122, *126*
Chevaugeon, J., 203, *210*
Chun, E. H. L., 174, 175, 176, *184*
Cieciura, S. J., 253, *289*
Cirarella, R. V., 177, *189*
Clark, A. J., 30, *36*
Clark, A. M., 223, *238*
Clarke, A. E., 132, *184*
Claus, G., 182, *186*
Cleland, R. E., 131, 144, *184*
Cleveland, L. R., 113, *125*
Cohen, S., 40, 43, *60*
Cohn, W. E., 27, *33*
Coleman, J. R., 108, *127*
Collinge, J. C., 172, *188*
Collins, F. M., 293, *321*
Comb, D., 177, *184*
Comfort, A., 294, 296, *321*
Commoner, B., 69, 70, *91*
Conklin, J. W., 302, *324*
Connell, D. I., 303, *321*
Conway, T. W., 23, 24, *33, 36*
Cook, J. R., 148, *184*
Coonradt, V. L., 198, *210*
Cooper, D. C., 228, *238*
Cooper, L. S., 228, *238*
Corbett, M. V., 69, *91*
Cornefert, F., 227, *238*
Cotlar, A. M., 318, *321*
Coulombre, A. J., 247, 248, *286, 289*
Coulombre, J. L., 247, *286*
Cowan, C. A., 169, 170, 173, *185*
Cowie, D. B., 99, *105*
Cox, E. C., 18, *34,* 181, *185*
Cox, R., *32*
Cramer, F., 32, *33*
Crick, F. H. C., 24, *33, 34*
Criddle, R. S., 42, *60*
Crouse, H. V., 119, 120, *125*
Cuany, R. L., 223, *240*

Curtis, H. J., 302, *321*
Cuzin, F., 118, *126*, 203, *210*

D

Dameron, F., 280, *286*
Daniel, V., 22, *33*
Danon, D., *36*
Darlington, C. D., 122, *125*
Darnell, J. E., 26, *37*
Das, C. C., 114, *125*
Davies, J. E., 18, *34*, 181, *185*
Davison, A. N., 301, *321*
Davison, P. F., 309, *323*
De, D. N., 116, *125*
Deeley, E. M., 122, *126*
de la Burde, R., 309, *321*
Delbrück, M., 209, *210*
De Maggio, A. E., 228, *238*, *241*
de Margerie-Hottinguer, H., 200, *211*
de Reuck, A. V. S., 317, *321*
Desai, I. D., 317, 319, *323*, *324*
De Salle, L., 176, 178, *188*
Dieckmann, M., 22, *37*
Dintzis, H. M., 31, *34*, *36*
Dixon, D. J., 44, *62*
Doberenz, A. R., 306, *324*
Dodson, J. W., 282, *286*
Doty, P., 31, *34*, *37*, *38*, 115, *127*, *129*, 177, *187*
Dounce, A. V., 114, 115, *128*
Dove, W. F., 97, *105*
Drahota, Z., 319, *324*
Dray, S., 41, 42, *60*, *62*, *63*
Drews, G., 45, *61*
Dublin, L. I., 298, *321*
DuBuy, H., 176, *185*
Dulbecco, R., 102, 103, 104, *105*, *106*
Dunn, D. B., 27, *35*, *37*
Dussoix, D., 28, 29, *32*, *34*

E

Eagle, H., 226, *238*, 250, 254, 278, *286*, *288*
Earle, W. R., 252, *290*
Easty, D. M., 277, *286*
Easty, G. C., 277, *286*
Ebringer, L., 181, *185*
Edelman, G. M., 39, 40, 42, 43, 44, 54, *60*, *61*, *63*
Edelman, M., 169, 170, 171, 172, 173, 175, *185*
Edmonds, M., 28, *33*
Edström, J. E., 116, *125*
Ehrmann, R. L., 273, *286*
Eigner, J., *34*
Eikenberry, E. F., 31, *34*
Eisen, H. N., 44, *61*
Eisenstadt, J., 141, 169, 173, 180, *184*, *185*
Eisenstein, R., 304, *321*
Ellis, H., 304, *321*
Ellison, S. A., 101, 102, *105*
Elsden, D. F., 304, *323*
Emery, G. C., 221, *238*
Englberger, F. M., 58, *62*
Ephrussi, B., 199, 200, 203, 204, 210, *210*, *211*, 227, *238*
Epstein, H. T., 132, 134, 136, 137, 138, 139, 140, 141, 142, 143, 145, 146, 147, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 169, 170, 171, 172, 173, 174, 175, *184*, *185*, *186*, *187*, *188*, *189*
Erwin, J., 141, *185*
Evans, A., 22, *35*
Evans, H. J., 111, 112, 119, *125*
Evans, W. R., *185*
Everett, G. A., 28, *35*
Everitt, A. V., 312, *323*

F

Fahey, J. L., 39, 41, 59, *61*, *64*
Falkow, S., 177, *187*, *189*
Fankhauser, G., 222, *239*
Fauvert, R., 41, *60*
Favelukes, G., *33*
Favelukes, S., *36*
Feinstein, A., 51, *61*
Fell, H. B., 245, *287*
Fernando, N. V. P., 305, *321*
Fessenden, J., 24, *34*
Fisher, H. W., 254, 277, *287*
Fisher-Fantuzzi, L., 99, *105*
Fitzgerald, R. G., 315, 316, *322*
Fjelde, A., 101, *105*
Flaks, J. G., 18, *34*, 181, *185*
Fleischman, J. B., 39, 40, 42, 43, 44, 52, *61*
Fleissner, E., 28, *33*, *34*

Fletcher, M. J., 301, *321*
Fougereau, D. V. M., 43, 54, *61*
Fox, J. E., 229, *238*
Frankel-Conrat, H., 69, 70, 79, 81, *91*, *93*, *94*
Franek, F., 53, *61*
Franklin, E. C., 39, 40, 41, *60*, *61*
Franklin, N. C., 30, *35*, 97, *105*
Franklin, R. M., 22, *37*
Fraps, G. S., 224, *239*
Freese, E., 79, *92*, 110, 111, 112, 118, *125*
Freese, E. B., 110, 111, 112, 118, *125*
Fresco, J. R., *34*, *37*
Friedman, S. M., 74, *92*
Fry, P., 308, *322*
Fudenberg, H. H., 41, 45, *61*
Fuerst, C. R., 98, *105*
Fuller, R. C., 140, *185*
Fuller, W., *34*
Furth, J. J., 20, 22, *34*, *35*

G

Gaines, 31, *33*
Gall, J. G., 116, 117, 118, *125*, *126*
Gally, J. A., 40, 54, *60*
Gardner, R. S., 31, *38*
Gaulden, M. E., 109, *126*
Gawlik, S. R., 154, 181, *188*, *189*
Gay, H., 114, 119, *125*, *126*
Geiduschek, E. P., 20, *34*, *38*
Gell, P. G. H., 51, *61*
German, J., 112, *126*
Gesteland, R. F., 22, *34*
Gey, G. O., 273, *286*
Giacomoni, D., 27, *34*
Gibbs, M., 140, *185*
Gibbs, S. P., 132, 134, 137, *185*
Gibor, A., 131, 147, 154, *185*, 237, *238*
Gierer, A., *34*, 69, 76, 77, 79, *92*, *93*
Gilbert, W., 21, *34*, *35*, 181, *185*
Ginoza, W., 69, *92*
Glagov, S., 310, *324*
Glinos, A. G., 301, *321*
Glowacki, E., *35*
Godman, G. C., 114, *125*
Gojdics, M., 144, *186*
Gold, M., 28, *34*
Goldberg, B., 270, *287*
Goldberg, I. H., 22, *35*
Goldsmith, T., 137, *187*
Goldstein, A., 31, *35*
Goldstein, L., 110, 117, *126*
Goldthwaite, D. A., 22, *37*
Golosow, N., 280, *287*
Gompertz, B., 296, *322*
Goodman, H. M., 27, *35*
Goodsell, S. F., 218, *238*
Gordon, A., 137, *187*
Gorini, L., 181, *185*
Grabar, P., 41, *60*
Granick, S., 131, 147, 154, *185*, *186*, 237, *238*
Grant, W. C., 301, *322*
Gray, R. A., 181, *186*
Graziosi, F., 20, *38*
Green, H., 270, *287*
Green, L., 176, *189*
Green, M. H., 21, *37*, 99, *105*
Greenblatt, C. L., 134, 142, *186*, *189*
Grell, S. M., 110, *126*
Grey, H. M., 41, *61*
Grindle, M., 201, *210*
Grobstein, C., 248, 249, 280, 281, 282, 283, 284, *287*, *288*, *289*
Gros, F., 21, 30, *32*, *35*, *36*
Gross, C. S., 315, 317, *324*
Gross, J., 304, 306, *322*, *323*
Gross, J. A., 140, *189*
Grubb, R., 41, *61*
Grunberg-Manago, 32, *36*
Guild, W., 177, *186*
Gurdon, J. B., 25, *33*, 35

H

Habel, K., 103, *104*, *105*
Haber, E., 58, *60*, *61*
Hall, B. D., 24, 25, *35*, *36*
Hall, W. T., 182, *186*
Halperin, W., 227, *238*
Ham, R. G., 278, *287*
Hamers-Casterman, C., 88, *92*
Hampar, B., 101, 102, *105*
Hanawalt, P. C., 110, *126*, 169, 1"3, *188*
Handler, P., 177, *186*
Harkness, M. L. R., 308, *322*
Harkness, R. D., 306, 308, 310, *322*

Harrington, W. F., 274, *287*
Harris, A. S., 318, *321*
Harris, H., 227, *238*
Harris, M., 277, *287*
Harrison, B. D., 85, *92*
Harrison, R. G., 276, *287*
Hart, R. G., 69, *92*
Hartman, P. E., 31, *32*
Hartmann, L., 41, *60*
Hartwell, L., 104, *105*
Haruna, I., 88, *94*
Haselkorn, R., 88, *93*, 176, 178, *188*
Hass, G. M., 304, *321*
Hauschka, S. D., 274, *287*
Hayashi, M., 20, 21, 30, *35*, *37*
Hayashi, M. N., 20, *35*
Hayes, D., 21, *36*
Hayes, F., 21, *36*
Hayflick, L., 226, 228, *238*, 293, *322*
Heikkinen, E., 308, *323*
Hellstrom, I., 103, *105*
Hemmens, W. F., 27, *35*
Hendlin, D., 181, *186*
Heremans, J. F., 40, *60*
Herrman, H., 250, *288*
Hershenov, B., 154, *187*
Hershey, A. D., 97, 99, *105*
Hiai, S., 109, *128*
Hiatt, H., 21, 22, *35*, *37*
Hidaka, Z., 84, *92*
Higa, A., 21, *35*
Hildebrandt, A. C., 227, 228, *238*, *241*
Hill, H., 146, 147, 148, *186*
Hillis, W. D., 278, *287*
Hiruki, C., 84, *92*
Hnilica, L. S., 115, *125*
Hobson, W., 299, *322*
Hoffmann-Berling, H., 66, *92*
Holland, I. B., 88, *94*
Holley, R. W., 28, *35*
Holoubek, V., 86, *92*
Holsten, R. D., 227, *240*
Holtermann, O. A., 101, *105*
Holtzer, H., 243, *290*
Hommes, F. A., 249, *287*, *288*
Hong, R., 44, 45, 47, 48, 49, *61*, *62*
Hopkins, J. W., 26, *36*
Horne, R. W., 66, *92*
Hotta, Y., 110, 111, 114, 122, *126*, *128*
Hottinguer, H., 203, *211*
Howze, G. B., 278, 279, *289*
Hufnagel, D. A., 173, *184*
Hunt, J., *32*
Hurwitz, J., 20, 22, 28, *34*, *35*
Hutner, S., 132, 181, *186*, *188*, *189*
Hsu, T. C., 101, 102, *105*, 111, 112, *126*
Hu, C. H., 236, *238*
Hughes-Schrader, S., 120, *126*
Hulett, H. R., 72, *92*
Humphrey, R. R., 222, *239*
Hungerford, D. A., 223, *240*
Huzisige, H., 134, 136, 137, *188*
Hyde, B. B., 119, *126*

I

Infanger, Sister Ann Martin, 198, *211*
Ingraham, L., 97, *105*
Inman, F. P., 49, 50, 51, *61*
Inoué, S., 119, *126*
Ishida, M. R., 174, *188*
Ito, M., 227, *239*
Ivanov, M. A., 221, *239*
Iwamura, T., 174, *186*

J

Jacob, F., 21, 29, *32*, *33*, *35*, *37*, 97, 98, 100, *104*, *105*, 118, *126*
Jagendorf, A. T., 141, *183*
Jagger, J., 148, 149, 150, *186*
Jakus, M. A., 272, *288*
James, R., 245, *290*
Jaquet, H., 44, *61*
Jaranowski, J., 222, *239*
Jeener, R., 88, *92*
Jinks, J. L., 201, 209, *210*, *211*
Jones, D. C. L., 302, *322*
Jones, O. W., 22, *37*
Joseph, K. T., 309, *322*
Joshi, J., 177, *186*
Justus, N., 219, 220, *239*

K

Kaesburg, P., 84, 85, *91*, *94*
Kahan, E., 22, *35*
Kahan, F. M., 22, *35*
Kahn, J. S., 180, *184*

Kallman, F., 249, 280, 281, 282, *288*
Kamen, M. D., 140, *188*
Kano-Sueoka, T., *35*
Kaplan, S., 84, *91*
Karush, F., 42, 43, 53, *64*
Kassanis, B., 85, *92*
Kastenbaum, M. A., 302, *324*
Kato, Y., 227, *239*
Katz, S., 177, *184*
Kaufmann, B. P., 114, 119, *125*, *126*
Keane, M., 181, *189*
Kehn, J. E., 40, 42, *63*
Kelly, D. E., 26, *37*
Kelner, A., 150, *186*
Kelus, A. S., 51, *61*
Kent, A. E., 227, *240*
Kerr, D. S., 22, *32*
Keynan, A., 21, *35*
Kihara, H., 222, *239*
Kimber, G., 218, *239*
Kimeldorf, D. J., 302, *322*
King, E. D., 119, *128*
King, T. J., 219, 221, 225, *238*, *239*
Kirk, J. T. O., 175, 181, *186*
Kirschbaum, J., 31, *35*
Kit, S., 176, *186*
Kitiyakara, A., 243, *288*
Kleczkowski, A., 88, *91*
Klein, G., 103, *105*
Klein, H. P., 141, *189*
Klug, A., 85, *91*
Knapp, E., 222, *239*
Knight, C. A., 88, *91*
Knopf, P. M., 23, *38*
Kock, A. L., 110, *127*
Kohn, R. R., 297, 299, 301, 306, 307, 308, 309, 310, 311, 312, 315, 316, 317, 318, *322*
Konar, R. N., 227, 230, 232, *239*
Konigsberg, I. R., 250, 252, 253, 254, 256, 258, 274, *287*, *288*
Konigsberg, N., 140, *184*
Konrad, 31, *33*
Korn, D., 98, *105*
Korngold, L., 41, *62*
Koshland, M. E., 58, *62*
Krakow, J. S., 20, *35*, *38*
Kreger, D. R., 132, *186*
Krinsky, N., 137, *187*
Krooth, R. S., 227, *239*
Krotkov, G., 140, *189*
Kruh, J., *36*
Kulonen, E., 308, *323*
Kunkel, H. G., 41, *61*, *62*
Kuntzel, H., 32, *33*
Kurland, C. G., 21, *35*
Kuwashima, S., 174, *186*

L

La Bella, F. S., 304, 309, *323*
La Cour, L., 122, *125*, *126*
Lal, N., 228, *239*
Lamfrom, H., *35*
Lamm, M. E., 40, 42, *63*
Lane, D., *36*
La Rue, C. D., 227, 229, *239*
Lascelles, J., 180, *184*
Lash, J. W., 249, *288*
Latarjet, R., 149, 150, *186*
Latham, H., 26, *37*
Laurell, A. B., 41, *61*
Lazzarini, R. A., 140, *187*
Leahy, J., 31, *33*
Leblond, C. P., 243, 244, 245, *288*
Leder, P., 19, *36*
Leff, J., 156, 168, 174, *187*
Lefort, C., 203, *210*
Lefort, M., 156, *187*
Le Grande, M. C., 301, *322*
Lengyel, P., 31, *38*
Lenoir, J., *63*
Lessell, I. M., 315, 317, *324*
Levinthal, C., 21, *35*
Levintow, L., 278, *288*
Levisohn, S., 176, *189*
Lewis, M. S., 304, 310, *323*
Lewis, S., 140, *187*
Licciardello, G., 97, *105*
Lichter, E. A., 41, *62*
Lieberman, I., 277, *288*
Likely, G. D., 252, *290*
Lima-de-Faria, A., 111, *126*
Lindenmayer, A., 237, *239*
Lindsay, W. G., 304, *323*
Lindstrom, E. W., 219, *239*
Lipari, R., 41, *62*

Lipkin, M., 301, *323*
Lipman, L. N., 44, *62*
Lipmann, F., 23, 24, *32, 33, 36, 38*
Liss, R., 134, 136, 150, 152, 154, 156, 158, 160, 162, 164, 166, *187*
Littauer, U. Z., 22, *33*
Littlefield, J. W., 27, *35*, 176, *184*
Loeb, T., 85, *92*
Loring, H. S., 72, *92*
Lotka, A. J., 298, *321*
Lowenstein, J., 40, *61*
Luck, D. J. L., 177, 178, *187*, 209, *211*
Luria, S. E., 30, *35*
Lyman, H., 136, 137, 145, 146, 147, 148, 149, 152, 172, *187, 188*

M

Maaløe, O., 110, *126*
Maas, W. K., 30, *36*
McCalla, D. R., 181, *187*
McCarthy, B. J., 21, 24, *33, 36*, 99, *105*
McCaughey, W. F., 306, *324*
McCay, C. M., 294, *323*
McClintock, B., 101, *105*
McConkey, E. H., 26, *36*
McDonald, M. R., 119, *126*
McElvain, N., 250, *288*
McGrath, R. A., 113, 114, *126*
McLeish, J., 114, *126*
Madison, J. T., 28, *35*
Mahoney, M., 203, *211*
Makela, O., 243, *288*
Malamy, M., 22, *35*
Mandel, H. G., 85, 88, *93*
Mandel, L. R., 28, *33, 36*
Mandel, M., 156, 168, 174, 176, *187, 189*
Mandelstam, J., 108, *127*
Mandy, W. J., 45, *62*
Mangelsdorf, P. C., 224, *239*
Mangold, O., 248, *288*
Mannik, M., 41, 56, *62*
Manton, I., 120, *126*
Mapes, M. O., 227, 228, *239, 240*
Marcou, D., 202, *211*
Marcus, P. I., 252, 253, *289*
Markham, R., 77, 85, *92, 93*
Marks, P. A., *36*
Markus, G., 45, *62*
Marler, E., 42, 49, *62*
Marmur, J., *34, 36, 37*, 115, *127*, 176, 177, *187, 189*
Marquisee, M., 28, *35*
Martin, G. R., 304, 310, *321, 323*
Marvin, D., 66, *92*
Masserman, R., 311, *324*
Mather, K., 113, *127*
Mattern, C., 176, *185*
Matthaei, J. H., 32, *33, 36*, 81, *93*
Matthews, R. E. F., 27, *33*, 85, 88, *93*
Matus, A. I., 85, 88, *93*
Mavioglu, H., 115, *125*
Mazia, D., 107, 109, 121, *127*
Mead, J., 310, *323*
Meeuse, B. J. D., 132, *186*
Mego, J. L., 181, *187*
Mellanby, E., 245, *287*
Mellon, A. D., 134, *189*
Meltzer, M., 40, *61*
Mercer, E. H., 245, *288*
Merrill, S. H., 28, *35*
Merryman, C., 39, *63*
Meselson, M., 21, *33*
Metzger, H., 55, 56, 57, *62*
Meyer, J. R., 219, 220, *239*
Michelson, A. M., 32, *36*
Michl, J., 277, *288, 289*
Midgley, J. F. M., 21, *36*
Migita, S., 41, *62*
Mikkonen, L., 308, *323*
Miksche, J. P., 223, *240*
Miles, C. P., 293, *323*
Miller, E. J., 304, *323*
Miller, R. S., 31, *38*
Mills, D., 88, *94*
Mishell, R., 39, *61*
Mitchell, H. K., 205, 206, *211*
Mitchell, M. B., 205, 206, *211*
Mitchison, J. M., 109, *129*
Mitra, J., 228, *239*
Mitsui, H., *37*
Mittermayer, C., 177, *184*
Miura, K. I., 84, *92*
Miura, T., 84, *92*
Moldave, K., 24, *34*
Monier, R., 21, *36*
Monod, J., 30, *35, 37*, 97, 100, *104, 105*

Monro, R., 24, *32*
Montagna, W., 245, *289*
Moohr, J. W., *34*
Moore, J. A., 219, 220, *240*
Moorhead, P. S., 293, *322*
Moriber, L. G., 154, *187*
Morris, A., *36*
Mortimer, R. K., 224, *240*
Moscona, A., 227, *240*
Moses, M. J., 108, 113, *127*
Movat, H. Z., 305, *321*
Muckenthaler, F. A., 111, *127*
Muller, H. J., 108, *127*
Mundry, K. W., 66, 68, 73, 74, 75, 76, 77, 78, 79, 80, 84, 85, 86, 87, 88, 89, *92*, *93*
Muntzing, A., 123, *127*
Muthukkaryppan, V., 247, *289*

N

Nagaraj, A. N., 90, *93*
Nagata, T., 98, *105*
Nagel, W., 319, *323*
Naithani, S. P., 223, *240*
Nakamoto, T., 20, 24, *34*, *36*
Nanney, D. L., 192, *211*
Naono, S., 21, 30, *32*, *36*
Nash, D., 112, 113, 118, *127*
Nass, M., 178, *187*
Nass, S., 178, *187*
Nataraja, K., 227, *239*
Nathans, D., 31, *36*
Naughton, M. A., 31, *36*
Nei, M., 236, *240*
Nelson, C. A., 42, 49, *62*
Neuberger, A., 306, *323*
Nezlin, R. S., 53, *61*
Nirenberg, M. W., 19, 22, 23, *33*, *36*, *37*, 81, *93*
Nishimura, M., 134, 136, 137, 140, *187*, *188*
Nisonoff, A., 41, 42, 44, 45, 46, 47, 48, 49, 50, 51, *60*, *61*, *62*, *63*
Nixon, H. L., 85, *92*
Noelken, M. E., 58, *62*
Noll, H., 23, *38*
Nossal, G. J. V., 243, *288*
Nussenzweig, R. S., 39, *63*
Nygaard, A. P., 25, *36*

O

Ochoa, S., 20, 31, *35*, *37*, 88, *91*, *94*
Oettgen, H. F., 39, *63*
Ogg, J. E., 224, *240*
Ohtaka, Y., 30, *37*
Oishi, M., 110, *127*
Olins, D. E., 39, 44, 54, *60*, *61*, *63*
Olsen, G. G., 312, *323*
Olsen, R. H., 224, *240*
Onoue, K., 54, 55, *63*
Osawa, S., *37*
Osserman, E. F., 40, *63*
Ostergren, G., 120, *127*
Otaka, E., *37*
Oudin, J., 41, *63*
Ovary, Z., 39, *60*
Ove, P., 277, *288*

P

Pachler, P. F., 110, *127*
Pain, R. H., 39, 40, 42, 43, 44, *61*, *63*
Palmer, J. L., 45, 46, 47, 49, *61*, *62*, *63*
Papaconstantinou, J., 247, *289*
Papajiannis, S., 304, *321*
Pardee, A. B., 29, *37*
Parsa, K., 311, *324*
Partanen, C. R., 225, *240*
Partridge, S. M., 304, *323*
Paul, G., 309, *323*
Paul, H. L., 66, *93*
Pauling, L., 58, *63*
Peacock, W. J., 120, *127*
Pearce, J. H., 44, *61*
Pearson, A. M., 317, *321*
Peckham, C., 309, *321*
Penswick, J. R., 28, *35*
Perini, F., 140, *188*
Perry, R. P., 26, *37*
Pfahl, D., 309, *323*
Philpott, G., 247, *289*
Piez, K., 254, *286*
Piez, K. A., 304, 310, *321*, *323*
Pinkus, H., 245, *289*
Pittenger, T. H., 206, *211*
Plaut, W., 112, 113, 118, *127*, 172, *188*
Pogo, A. O., 140, 181, *184*, *188*
Pogo, B. G. T., 181, *188*
Pollock, M. R., 108, *127*

Poole, J. P., 278, *289*
Porter, R. R., 39, 40, 42, 43, 44, 52, *60*, *61*, *63*
Poulik, M. D., 39, 40, 42, 43, *60*
Preiss, J., 22, *37*
Prensky, W., 113, *129*
Prescott, D. M., 107, 110, 114, 115, 116, 117, 121, *125*, *127*, *128*
Press, E. M., 39, 44, 52, *61*, *63*
Pressman, D., 54, 55, 56, *63*
Price, C. A., 141, *188*
Pringsheim, E., 145, 181, *188*
Pringsheim, O., 181, *188*
Proctor, H. R., 306, *323*
Provasoli, L., 132, 181, *186*, *188*
Puck, T. T., 252, 253, 254, 277, *287*, *289*
Putnam, F. W., 41, 42, *60*, *62*, *63*

R

Rabinowitz, M., 22, *35*, 176, 178, *188*
Radzimski, G., 56, *63*
Ralph, R. K., 85, 88, *93*
Ramanis, Z., 205, *211*
Randall, J., 114, 115, *128*
Rapp, R., 101, 102, *105*
Rappaport, C., 278, 279, *289*
Rappaport, H. P., 278, *289*
Raven, P. H., 237, *240*
Ray, D. S., 169, 173, *188*
Ray, W. J., Jr., 24,
Read, J., 118, *128*
Reddi, K. K., 90, *93*
Rees, M. W., 85, *93*
Reich, E., 22, *35*, *37*, 177, 178, *187*, 209 *211*
Reichmann, M. E., 85, *93*
Reisfeld, R. A., 42, *63*
Revel, M., 22, *37*
Rhoades, M. M., 122, 123, *128*, 131, 144, *188*
Rich, A., 23, 27, 31, *34*, *35*, *38*, 174, 175, *184*
Rier, J. P., 228, *241*
Rifkind, R., *36*
Rigby, B. J., 308, 312, *323*
Riker, A. J., 227, 228, *238*, *241*
Riley, F., 176, *185*
Riley, R., 218, *239*
Ris, H., 119, 122, *128*, 172, *188*
Risebrough, R. W., 21, *35*
Risser, P. G., 228, *240*
Ritossa, F. M., 25, *37*
Rivat, L., 41, *63*
Rivera-Velez, J., 310, *322*
Rivers, M. M., 45, *62*
Rizet, G., 202, 209, *211*
Ro, T. S., 115, *125*
Roberts, R. B., *36*
Roger, M., 66, *94*
Roholt, O. A., 54, 55, 56, *63*
Rollerson, E. R., 306, 307, 308, 309, 310, *322*
Roman, A., 31, *35*
Roman, H., 200, *211*
Ropartz, C., 41, *63*
Roper, A., 201, *211*
Rosen, W. G., 154, 181, *188*, *189*
Rosenberg, A., 141, *188*
Rosenberg, B. H., 109, 116, *125*
Rosenberg, M. D., 277, *289*
Rownd, R., 177, *187*
Rubin, A. C., 309, *323*
Rudkin, G. T., 223, *240*
Rudzinska, M. A., 293, *323*
Rusch, H., 177, *184*
Russell, G. E., 66, *92*, *93*
Russell, E., *33*
Rutter, W. J., 249, 280, 283, *289*

S

Sacher, G. A., 312, *323*
Sager, R., 136, 137, 174, *188*, 205, *211*
Sagik, B. P., 21, *37*
Salas, M., 31, *37*
Samuels, H. H., 20, *38*
Sanadi, D. R., 301, *321*
Sanford, K. K., 252, *290*
San Pietro, A., 140, *187*
Sarnat, M., 20, *38*
Sato, G., 277, *287*
Savage, J. K. R., 120, *125*
Sawant, P. L., 319, *323*
Sax, K., 119, *128*
Schaechter, M., 110, *127*
Schairer, L. A., 223, *240*
Schatz, A., 181, *188*

Schaub, M. C., 308, *323*
Schecroun, J., 209, *211*
Scher, S., 141, 172, *183*, *188*
Scherrer, K., 26, *37*
Schiff, J. A., 132, 134, 136, 137, 138, 139, 140, 141, 142, 143, 145, 146, 147, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 169, 170, 171, 172, 173, 174, 175, *184*, *185*, *186*, *187*, *188*, *189*
Schildkraut, C., *34*, 176, 177, *187*, *189*
Schlessinger, D., *36*
Schlueter, R. J., 309, *323*
Schmid, W., 111, 112, *126*
Schmitt, F. O., 304, 309, *323*, *324*
Schramm, G., 69, 75, *92*, *93*
Schultz, J., 223, *240*
Schuster, H., 75, 79, 80, *93*
Schweet, R., 22, 24, 31, *32*, *33*, *36*, *38*
Scott, D., 111, 112, *125*
Sehgal, N., 230, *241*
Sela, M., 58, *60*
Selman, G. G., 221, *240*
Shaeffer, J., 24, *32*
Shapanka, R., 58, *62*
Sharpe, H. S., 200, *211*
Shatkin, A. J., 22, *37*
Shearer, G. B., 70, *91*
Shibko, S., 317, *324*
Shimura, Y., 176, *187*
Shipp, W., 88, *93*
Shock, N. W., 299, *324*
Sibatani, A., 109, *128*
Siegel, A., 78, 88, *93*
Siegesmund, K. A., 154, *189*
Simon, E. H., 117, *128*
Simon, E. J., 315, 317, *324*
Sinclair, J., 176, 178, *188*
Sinex, F. M., 308, *324*
Singer, B., 70, *93*
Singer, M. F., 22, *37*
Singer, S. J., 55, 57, *62*
Singh, E. J., 115, *125*
Sjögren, H. O., 103, *105*
Slack, H. G. B., 306, 315, 317, *323*, *324*
Small, P. A., 40, 42, *63*
Smillie, R. M., 140, 172, 180, *185*, *187*, *189*
Smith, J. D., 27, *37*
Smith, J. H. C., 134, 180, *189*
Smith, M., 176, *189*
Smith, M. A., 31, *37*
Smith, R. E., 223, *238*
Smith, W., 86, *93*
Smith, W. M., 31, *37*
Smith-Sonneborn, J. E., 176, *189*
Sobel, H., 280, *290*, 311, *324*
Sonneborn, T. M., 192, *211*
Sorieul, S., 227, *238*
Sparrow, A. H., *129*, 223, *240*
Speakman, P., 309, *323*
Speigelman, M., 298, *321*
Spemann, H., 248, 279, *288*, *290*
Speyer, J. F., 31, *38*
Spiegelman, S., 20, 21, 24, 25, 26, 27, 30, *34*, *35*, *37*, *38*, 88, *94*
Spink, J. M., 314, *324*
Spotts, C. R., 181, *189*
Srb, A. M., 193, 197, 198, *211*
Srinivasan, P. R., 26, 28, *37*
Stanier, R. Y., 181, *189*
Stanley, W. M., Jr., 31, *37*
Staples, R., 66, *91*
Starbuck, N. C., 115, *125*
Starr, J. L., 22, 28, *37*
Stebbins, G. L., 107, 123, *128*
Steele, W. J., 115, *125*
Steere, R. L., 66, *94*
Steeves, T. A., 228, *241*
Steffensen, D. M., 118, *128*
Steinberg, M. S., 279, *290*
Stemke, G. W., 51, *63*
Stent, G. S., 27, 31, *33*, *37*, *38*
Stern, A. I., 136, 137, 139, 141, 154, 156, *187*, *189*
Stern, H., 109, 110, 111, 114, 121, 122, 123, *126*, *128*
Stevens, A., 20, *38*
Steward, F. C., 227, 228, *239*, *240*
Stich, H. F., 101, 102, *105*
Stockdale, F. E., 243, *290*
Stockwell, R. A., 311, *324*
Stodolsky, M., 20, *38*
Stoeckenius, W., 66, *94*
Stone, B. A., 132, *184*
Stone, G. E., 110, *128*
Stone, L. S., 248, *290*
Stow, I., 223, *240*

Strehler, B. L., 294, 301, *324*
Stretton, A. O. W., 84, *91*
Strode, C., 70, *91*
Strudel, G., 249, *290*
Stubblefield, E., 111, 112, 122, *126*, *128*
Subtelny, S., 219, 221, 225, *240*
Sueoka, N., 23, *38*, 110, *127*, 176, *189*
Sugiyama, T., 70, *94*
Suomalainen, E., 218, *240*
Sundararajan, T. A., 31, *38*
Swanson, C. P., 119, *128*
Swift, H., 176, 178, *188*
Symons, R. H., 85, *93*

T

Takata, C., 247, *290*
Takatsuki, K., 40, *63*
Tanford, C., 42, 49, 58, *60*, *62*, *64*
Tappel, A. L., 317, 319, *323*, *324*
Tatum, E. L., 22, *37*
Taylor, A. C., 277, *290*
Taylor, A. L., 100, *105*
Taylor, C. W., 115, *125*
Taylor, J. H., 109, 113, 117, *127*, *128*
Terry, W. D., 41, *64*
Thach, R. E., 31, *38*
Theil, E. C., *38*
Thomas, J., 304, *323*
Thompson, H. J., 237, *240*
Thompson, R. C., 306, *324*
Thorbecke, G. J., 44, *62*
Thrasher, J. P., 318, *321*
Ting, R. C., 103, *105*
Tissieres, A., 205, *211*
Tocchini-Valentini, G. P., 20, *38*
Tominaga, K., 41, *60*
Tootle, M., 250, *288*
Torrey, J. G., 228, *240*
Trim, A. R., 66, *92*
Trosko, J., 115, 116, 120, *128*
Ts'o, P., 114, *125*
Tsugita, A., 79, 81, *94*
Tsunewaki, K., 222, *239*
Tulecke, W., 229, 230, 233, *240*, *241*

U

Umana, R., 114, 115, *128*
Updike, S., 114, 115, *128*
Upton, A. C., 302, *324*
Utsumi, S., 42, 43, 53, *64*

V

Valentine, R. C., 66, *94*
van Erkel, G. A., 305, *321*
Van Holde, K. E., 45, 46, *63*
van Leeuwen, G., 249, *287*
Vasil, I. K., 227, *241*
Vasil, V., 227, *241*
Vaughan, M. H., Jr., 174, 175, *184*
Veis, A., 309, *321*, *323*
Vermeulen, C. W., 25, *38*
Verzár, F., 306, *324*
Vinograd, J., 103, *106*
Vogel, H. J., 176, *187*
Vogt, M., 102, 103, 104, *105*, *106*
Voigt-Koehne, L., 88, *94*
von Borstel, R. C., 224, *240*
von Ehrenstein, G., 24, *33*, *36*, *38*
von Hippel, P. H., 274, *287*
von Wettstein, D., 90, *94*, 136, 137, 142, *189*

W

Wahba, A. J., 31, *37*, *38*
Wakonig, T., 120, *127*
Walen, K. H., 117, *129*
Walker, P. M. B., 109, *129*
Ward, M., 227, *241*
Warner, J. R., 23, *38*
Warner, R. C., 20, *38*, 88, *91*
Watanabe, I., 84, *92*
Watson, J. D., 21, *35*
Watts-Tobin, R. J., 24, *33*
Weil, R., 103, *106*
Weinstein, I. B., 74, *92*, *94*
Weisblum, B., 24, *33*, *38*
Weiss, M. C., 227, *238*
Weiss, P., 245, *290*
Weiss, S. B., 20, *34*, *36*, *38*
Weissbach, A., 98, *105*
Weissmann, C., 88, *91*, *94*
Wessells, N. K., 243, 249, 280, 282, 283, *289*, *290*
Westergaard, M., 124, *129*
Wetherell, D. F., 227, *238*

Wetmore, R. H., 228, *241*
Wettstein, F. O., 23, *38*
White, F. H., Jr., 58, *60*, *64*
White, J. R., 18, *34*, 181, *185*
Whitney, P. L., 58, *60*, *64*
Whittier, D. P., 228, *241*
Wiesmeyer, H., 100, *106*
Wildman, S. G., 88, *93*
Wilkie, D., 203, 204, 209, *211*
Williams, R. C., 66, *94*
Williams, R. P., 293, *321*
Williamson, A. R., 31, *38*
Willig, F., 319, *323*
Willson, C., *36*
Wilson, G. B., 122, *129*
Wilson, J. A., 307, *323*
Wimber, D. E., 113, *129*
Winocour, E., 103, *106*
Wissler, F. C., 44, 45, *62*
Witting, M. L., 18, *34*
Wittmann, H. G., 19, *38*, 73, 79, 81, 83, *93*, *94*
Wittmann-Liebold, B., 19, *38*
Woernley, D. L., 44, *62*
Woese, C. R., *38*
Wopy, L., 57, *62*
Wohlhieter, J. A., 177, *189*
Wolff, S., 115, 116, 120, 121, *128*, *129*
Wolinsky, H., 310, *324*
Wolken, J. J., 132, 134, 140, *189*
Wollman, E. L., 98, *105*
Woodruff, M., 140, *187*
Wright, E. A., 314, *324*
Wunderlich, J., 39, *61*
Wyckoff, R. W. G., 306, *324*

Y

Yamada, T., 247, *290*
Yamane, T., 23, *38*
Yamazaki, H., 85, *94*
Yankofsky, S. A., 25, 26, *38*
Yanofsky, C., 19, *38*, 97, *105*
Yarmolinsky, M. B., 100, *106*
Yoshikawa, H., 110, *127*
Yotsuyanagi, Y., 205, *211*
Young, R. W., 243, *290*
Yu, S. Y., 304, *324*

Z

Zahalsky, A. C., 181, *189*
Zak, R., 319, *324*
Zalkin, H., 317, *324*
Zamenhof, S., *38*
Zamir, A., 28, *35*
Zech, H., 88, 89, 90, *94*
Zeuthen, E., 110, 121, *129*
Zilliken, F., 249, *287*, *288*
Zinder, N. D., 54, *60*, 66, 85, *92*, *94*
Zubay, G., 115, *129*
Zwilling, E., 294, *324*

Subject Index

A

Adrenal, 244
Aegilops, 222
Aging
  as consequence of growth cessation, 291
  chromosomal aberrations and, 302
  definition of, 292, 319
  growth and, 294
  ionizing radiation and, 302
  of cells, 313
  role of cells in, 299
    of connective tissue in, 303
  thermal denaturation of collagen and, 308
Aging population, characteristics of, 295
Algal chloroplasts, 132
Allopolyploids, 218
Ameba, migratory proteins in, 110
Amino acid replacements, 81
δ-Aminolevulinic acid, 154
Δ-Aminolevulinic synthetase, 180
β-Aminopropionitrile, 310, 313
Aminotriazole, 141
5-Aminouracil, 120
Androgenetic haploids, 222
Angiosperms, 134
cis-Antheraxanthin, 137
trans-Antheraxanthin, 137
Antibody
  activity
    in isolated heavy chains, 52
    of recombined heavy and light chains, 53
  light chains in active site of, 57
Antibodies
  reconstitution of, 54
  specificity and amino acid sequence, 57
    structural basis of, 39
  synthesis, 3
Anticodon, 18, 19
Antimycin A, 254
Antirrhinum, 229, 230, 237
Apogamy, 228
Arteriosclerosis, 297
Aspergillus
  alba variant of, 203, 204
  mycelial variants of, 201
  red variant of, 201
Autopolyploids, 218
Autosynthesis, 3
Auxin, 229
Azathymine, 172

B

Bacterial chromosome, 110
Bacteriophage λ, 96, 99
Balanus, 176
Barrage phenomenon in *Podospora*, 209
Basal body, 132
Basal cell of the epidermis, 245
Bence Jones proteins, 40, 59
Beta vulgaris, 175
Blastocladiella, 177
bobbed, 25, 26, 27
Broad bean mottle virus, 71
5-Bromodeoxyuridine, 102
Bromosuccinimide, 79
Bromouracil, 172

C

Ca^{++}, 277
Cancer, 176, 297
Cancer-producing viruses, 96
Carbon dioxide fixation, kinetics of appearance of, 139
Carcinogenic adenovirus, 102
β-Carotene, 137
Carotenoid, 137
  kinetics of appearance of, 139
Caryogamy, 124
Cell adhesions, 278
  monovalent cations and, 278
Cell attachment, 277
Cell culture and macromolecules, 276
Cell division, 243
Cell populations, static, expanding, or renewing, 244
Cell transformation, 102
Cell type, interactions in reproduction of cell, 243
Cells
  aging of, 313
  cytodifferentiation, in culture, 249

Cellular regulation, 12
Chemomutagenesis, 79
Chick, 176
  embryo, thigh muscle, 251
  leg muscle, 250
Chicorium, 227
Chinese hamster cells, 102
Chlamydomonas, 134, 136, 156, 168, 174, 205
Chlorella, 134, 136, 174
Chlorophyll
  absorption spectra of, 235
  kinetics of appearance of, 139
  light requirement for formation of, 134
Chlorophyll *a*, 137
Chlorophyll *b*, 137
Chloroplast(s), 131, 132
  continuity in *Euglena,* 131
    replicative aspect of, 143
  development, 136
    control mechanisms for, 178
    iron in, 141
    model for control of, 179
    ribosomes in, 140
    RNA during, 140
  developmental physiology, 137
  division, cytological evidence for, 144
  DNA in, 156, 178
  evolution, 182
  light requirement for formation of, 134
Chromatid, 121
  aberrations, 119, 120
  breaks, 101
Chromosomal aberrations and aging, 302
Chromosomal components, formation of, 114
Chromosomal RNA, 116
Chromosome(s)
  contraction of, 112
  reproduction, 116
    consequences of, 121
    in mitosis and meiosis, 107
Chromosomin, 115
Chrysanthemum, 223
Cistron, functional separation, 83
Codons, 18, 19
Colchicine, 120
Collagen, 272, 273, 274, 278, 280, 282, 283, 304, 306, 307, 309
  cross linking, 308
    chemistry of, 309
  deposition of, 270
  differentiation and, 279
  substratum, effect of upon development of muscle clones, 275
  thermal denaturation of aging and, 308
Colony types, comparison between unconditioned and conditioned medium in development of, 257
Complementation, 206
Conditioned medium
  development of muscle clones in, 253
  hypothetical role of, 273
Conditioning
  biological parameters of, 256
  collagen synthesis and, 268
Conditioning petri plate surface, 262
Conditioning process of culture medium, 254
Contraction of chromosomes, 122
Conversion, virus-induced, 96
Cornea, 272
Cortical inheritance, 192
Crepis, 237
Crithidia, 176
Cryptoxanthin, 137
Cupressus, 229, 230
Curvularia, 203
Cyanophora, 183
Cycas, 227
Cysteine tRNA, 24
Cytidylic acid triplet, deamination of, 82
Cytochrome, 140, 154
Cytodifferentiation, 243
  in cell culture, 249
  population density and, 252
Cytokinesis, 121
Cytokinin, 229
Cytoplasmic heredity, 191

D

Datura, 218
Descemet's membrane, 272
Differentiation, 245
  collagen and, 279

Differentiative function, stability of in culture, 250
Digestive tract, 244
6-Dimethylaminopurine, 27
Dimethyl sulfate, 79
reaction of TMV RNA with, 80
Diploid(s), 218
cell, doubling, 226
plant cells, 227
Diploidy haploidy, 217
Disc, kinetics of formation of, 138
DNA
from *Euglena,* 168, 169, 173
nongenetic, 26
of chloroplast, 156, 178
of chloroplast-containing species, 174
of *Euglena* mutants, 172
replication of, 109
satellite, 176
synthesis
in progenitor cells, 243
time, 111
Drosophila, 25, 26, 27, 111, 112, 113, 123, 221, 223
Duplicase, 19

E

Echinenone, 137
Elastin, 303
Embryonic induction, 246, 248
experimental approaches to, 248
Endoplasmic reticulum, 132
Endopolyploidy, 121
Ephedra, 230, 232
Epidermal-dermal relationship, 282
Epidermis, 244
basal cell of, 245
keratinizing, 245
Epilobium, 218
Epitheliomesenchymal interacting systems, 280, 282
Ergosterol, 141
Escherichia coli, 18, 22, 25, 26, 27, 28, 30, 81, 83, 96, 99, 100, 103, 149, 150, 182, 214, 224, 225
Euglena, 7, 131, 144, 168, 173
DNA from, 169
mutants, 172
cells, cytology of, 152
olive-green, 156, 160, 162
pale-green, 158
white, 150, 152, 154, 156
yellow, 150, 156, 164, 166
plastids of, 158
proplastids of, 158
in dark-grown, 136
Euglena gracilis var. *bacillaris,* 131
Euglena gracilis Z strain, 132
Euglenanone, 137
Euploids, 218
Expanding cell population, static, or renewing, 244
Extrachromosomal heredity
evidence for, 193
experimental detection of, 196
in fungi, 191
Extrachromosomal variability
origins of, 203

F

Fc
effect of pH on sedimentation of, 50
molecular weights of, 51
reversible dissociation of fragment of, 49
Fibroblastic clones grown in conditioned and unconditioned medium, 264
Fibroblast and muscle colonies
subclones of, 261
Fibroblast cells, 273, 280
discoid colonies of, 254
growth of, 270
Fibroblast monolayer, electron micrograph of, 271
Fluorouracil, 180, 181
Fungi, extrachromosomal heredity in, 191

G

gal, 30
Gametophyte
female, 233
male, 229
Gamma crystalline, 247
Gene action system, 17
genetic basis of, 24
Gene transcription and translation of, 17
Genes, *ur,* 17
components specified by, 19

Genetic code, TMV mutants and, 79
Genetic interactions between organelles
  hypothetical schemes for, 143
Ginkgo, 217, 229, 230, 232, 234, 235, 236
  development of, 231
γG-Globulin
  complementation polypeptide subunits of, 51
  half-molecules of, 48
    dissociation of (rabbit) into, 45
    univalence of, 48
  schlieren patterns of, 46
  spontaneous reassociation of half-molecule subunits, 47
Golgi body, 132
Gossypium, 218
Grasshopper, 111, 113
  neuroblasts, 109
Growth
  aging and, 294
  cessation and aging, 291
  temperature and replication errors, 73
Guinea pig, 39, 54, 176
Gymnosperms, 134
Gynogenetic diploids, 220

H

Habrobracon, 223
Hadacidin, 181
Hair, 245
Half-chromatid aberrations, 119, 120
Halobacterium, 177
Hamster cell, 111
Hamster cheek pouch, electron micrograph of capillary of, 305
Haploid cotton plant, 220
Haploid syndrome, 219, 220, 222
Haploid tissue cultures of higher plants, 229
Haploid tissues, characteristics of, 232
Haploids, 218
Haploidy, induction of, 221
Haplopappus, 228
Healing enzyme, 113
Heavy chains, antibody activity in isolated, 52
Hematopoietic tissue, 244
Heme, 154
Heredity, extrachromosomal in fungi, 91
Herpes virus, 102
Heterosynthesis, 3
Histones, 114
Holochrome particle, 134
Host cells, interactions with plant viruses, 65
Human cell, 111
Hyacinthus, 223
Hydroxyechinenone, 137
Hydroxylamine, 79, 102
Hydroxylamine-sensitive bonds, 309
6-Hydroxy-2-methyldiaminopurine, 27
Hypertension, 297

I

Immunoglobulin(s), 39
  properties of, 40
  structural model of, 43
Infectivity, integrity of viral RNA and, 69
Interaction between chromosomal mutants, 205
iojap, 144
Ionizing radiation and aging, 302
Iron, in chloroplast development, 141

K

K^+, 24, 278
Karyokinesis, 111
Keratin synthesis, 244
Keratinizing epidermis, 245
Kidney, 244, 280
  pancreatic acinar cells of, 244
  proximal convoluted tubule cells of, 244
Killer phenomenon, 192

L

lac, 30
Lactate production, 315
  in leukocytes, 316
Lactuca, 227
Lamella, 132, 137, 141, 142, 154
  kinetics of formation of, 138
Lampbrush chromosomes, 117, 118
Lathyrism, 313
Lathyrogenic agent, 310
Leishmania, 176
Lens, growth and development of, 247

Leukocytes
lactate production in, 316
properties of, 316
succinic dehydrogenase in, 316
Li^+, 24
Lily microspores, 111
α-Linolenic acid, 141
little, 210
little yeast, 209
Llaveiella, 120
Lung, 280
Lycopodium, 228
Lymphatic malignancies, 40
Lyon's effect, 111
Lysogenization, 97, 102
Lysogeny, significance of, 99
Lysosomal enzymes, 317
Lysosomes, 318, 319

M

Macromolecules and cell culture, 276
Malignancy, 297
Malignant neoplasms, 312
Mantid, 113
Meiosis chromosome, 107
Melilotus, 222
Merozygotes, 30
Messenger RNA (mRNA), 18, 21, 142
Methionine, 28
2-Methyladenine, 27
6-Methylaminopurine, 27
1-Methylguanine, 27
Mexican axolotl, 222
Mg^{++}, 23
Microspectrophotometry of TMV infected cells, 88
Migratory proteins in ameba, 110
Minimal infectious genetic information, 84
Minutes, 27
Mitosis chromosome, 107
Modulations, 245
Molecular regulation, 8
Mormoniella, 224
Mortality from selected causes by age, 297
Mosquito, 110
Mosses, 228
Mouse, 39, 176
embryos, epithelium from lens, 247
Mucoid-secreting epithelium, 245
Mucopolysaccharides, 249, 283
Multiple myeloma, 40
Muscle
chick leg, 250, 251
fibroblast colonies subclones of, 261
skeletal, 250
Muscle cathepsin, 317
Muscle clones
development of, 253
effect of conditioned medium upon, 267
on reconstituted collagen, 276
Muscular dystrophy, 317
Mutant phenotype, suppression of, 206
Mutation and temperature, 75
Mutual exclusion, 77
Myeloma proteins, 59
Myosin, 317
Myosin-degrading enzyme, 317
Myotubes, 251, 252, 253

N

Na^+, 278
Naegleria, 176
Neoguanylate, 27
Neoxanthin, 137
Neurons, 244
death of, 314
Neurospora, 124, 177, 178, 193, 194, 195, 196, 203
poky variant of, 205, 206
Neutrophils, 315
NH_4^+, 24
Nicotiana, 218, 237
Nitrous acid, 79, 81
Nongenetic DNA, 26
Nuclear-cytoplasmic ratio, 224
Nuclear pore, 132
Nucleic acids, specific alteration of, 27
Nucleolus, behavior of, in virus-infected cells, 90
Nucleotide
chemomutagenesis, 75
nitrous acid and, 75
replacement, 75

O

Ochromonas, 134, 175
Oenothera, 144, 178, 218, 229
O-Methyl threonine, 181
Organelle continuity, 131

P

Pancreas, 244, 280
tissue interactions in developing, 280
Pancreatic epithelial cells, electron micrograph of, 281
Papain, 44
Paramecium, 176, 214
Paramylum, 132
Pellicle, 132
Pepsin, 44
Peptide polymerase, 19, 24
Pestalozzia, 203
Petri plate surface
conditioning, 262
effect of pretreating, 265
Phenotypic effects, 204
Pheophytin, 142
Phospholipids in chromosomes, 122
Photoreactivation, 147, 148, 149, 152
Photosynthesis, 137
Photosynthetic oxygen, kinetics of appearance of, 139
Physarum, 177
Pinocytosis, 86
Pinocytotic vesicle, 132
Pituitary, 280
Plant viruses
interactions with host cells, 65
structure and infectivity of, 66
Plastids, 158, 162, 164, 166
Pleural epithelium, 244
Podospora, 202, 203
Podospora senescent variant of, 202
poky Neurospora, 209
Polio virus, 96
Pollen, 229, 232
development *in vitro*, 230
Polycistronic messenger, 83
Polycistronic mRNA, 21
Polyoma virus, 102, 103, 104
Polyploids, 218
Polytene, 119
chromosomes, 117
Polyteny, 121
Polytoma, 156, 168, 174
Polyvalent autocatalysis, 108
Population density and cytodifferentiation, 252
Potato virus X, photoreaction of, 88
Progenitor cell, 244
DNA synthesis in, 243
Prolamellar body, 136
Prophage, 97
genetic map, 97
Proplastid, 132, 142, 145, 154, 158, 164, 166, 179
condition, reversal to, 141
in dark-grown *Euglena*, 136
membrane, 137
Proteins, 40
synthesis of, 139
turnover, studies of, in atrophying cells, 315
Proteolipids, 301
Proteus, 177
Protochlorophyll, 134, 152, 179, 180
Protoporphyrin, 154
Pseudouridine, 27
Pteridium, 228
Pteris, 228
Putrescine, 278
Pyrenoid, 137

R

Rabbit, 41, 54
Rana, 219, 224, 225
androgenetic homozygous larvae of, 221
Raney nickel, 24
Ranunculus, 227
Rat, 244
Replication
errors and growth temperature, 73
of DNA, 109
Replicons, 113
Reproduction
cell type
cell interactions in, 243
tissue interactions in, 243

mechanisms of cellular, 6
molecular, of, 2
subcellular, 4
Reticuloendothelial system, 244
Ribosomal components, 18
Ribosome during chloroplast development, 140
RNA
during chloroplast development, 140
messenger (mRNA), 18, 21
polymerase, 18, 20
ribosomal (rRNA), 18
template (tRNA), 180
transfer, 18

S

Saccharomyces, variants of, 199
Salivary gland, 280
Salmine, 277
Salmon, 176
Satellite DNA, 169, 170, 171, 172
Schlieren patterns, 46, 48
of half-molecules of γG-globulin, 48
Sebaceous glands, 245
Sedum, 223
Seminiferous tubules, 244
senescent, of *Podospora* variant, 202
Serratia, 30, 177
Sheep, 54
Siredon, 222
Skeletal muscle, 250
cells, fibrous colonies of, 254, 255
Specificity and antibody and amino acid sequence, 57
S period, 109, 110, 111
Spinacia, 175
Spinal cord, 249
Spinal cord-chondrogenic interaction, 249
Spongioblasts, 244
G_1 stage, 110
G_2 stage, 110
Static cell population, 244
Stem cell
differential division of, 245
differentiation of, 246
cellular environment and, 246
regulation of, 245
Stem cell line, 244
Stratified squamous epithelium, 245
Subcellular regulation, 10
Succinate dehydrogenase, 315
Succinic dehydrogenase in leukocytes, 316
Sugar beet yellows virus, 66, 67, 68
Suppression of mutant phenotype, 206
SV40, 102
Synapsis, 123

T

Taxus, 230, 232, 233
T2 phage, 110
Temperature and mutation, 75
Template RNA (tRNA), 180
methyl-poor, 28
Tendon fibers, Young's modulus of, 307
Terminal nucleotide sequence of TMV RNA, 70
Tetrahymena, 109, 110, 114, 121
Tetraploids, 218
Thermal denaturation of collagen and aging, 308
Thymine, 27
Thymus, 280
Thyroid, follicular cells of, 244
Tissue, interactions in reproduction of cell type, 243
TMV, mutation of strain *vulgare*, 74
TMV RNA, terminal nucleotide sequence of, 70
Tobacco mosaic virus (TMV), 66, 67, *see also* TMV
Tobacco necrosis satellite virus, 85
Tobacco rattle virus, 66, 67, 85
Tocophrya, 293
Todea, 120, 228
Torreya, 230, 232, 233
Totipotency of diploid plant cells, 227
TPN-transhydrogenase, 140
TPN-triose phosphate dehydrogenase, 130
Tradescantia, 119
Transcribing enzyme, *see* RNA polymerase
Transcriptase, 19
Transcription, 20
Transfer enzymes, 19
Transfer RNA (tRNA), 18

Transformation, 96
Translation, 23
Triplet hypothesis, 81
Triploids, 218, 224
Triticum, 218, 222
Triton, 225
Triturus, 118, 221
Trollein, 137

U

Ultraviolet inactivation, 147, 148, 149
Ultraviolet-sensitive chromophore, 147
Ultraviolet-sensitive sites, 147
Unwinding, 112, 118

V

Vegetative genetic map, 97
Vicia, 111, 112, 115, 120, 175
Viral DNA, attachment and insertation of, 99
Virus
  broad bean mottle, 71
  coat protein, 86
  infection
    consequences for genetic material of
      bacterial cells, 96
      animal cells, 101
  interaction with host genetic material, 95
  mutation, 72
  particles, minimal infectious length of, 66
  polio, 96
  replication, cellular events in, 86
Virus-cell complex, 95, 96
Vitamin A, 245
  in cord endosperm, 224

W

Waldenstrom's macroglobulinemia, 59
Wheat streak mosaic virus, 66, 67

X

Xenopus, 25, 26

Y

Yeast
  variants, *little*, of, 199, 200
    suppressive of, 200

Z

Zamia, 227
Zea, 218, 222, 237
Zeaxanthin, 137