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ADVANCES IN CELL AND MOLECULAR BIOLOGY

Volume 1

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Edited by E. J. DuPraw

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This volume is dedicated to Sophia DuPraw
who typed the manuscript and gave birth to the editor,
not necessarily in that order

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PREFACE

Advances in Cell and Molecular Biology has been designed as a research-oriented serial, which will provide scholarly yet readable communication among those interested in the cell–molecular sciences. In particular, we hope to provide a meeting ground between biochemically and ultrastructurally oriented researchers. Each article is divided about equally between original information from the author's own laboratory and a readable review of its importance relative to research from other laboratories.

Since this first volume keynotes the series, we are particularly fortunate to have brought together a group of authors, every one of whom represents a laboratory ranked among the best in the world in its particular specialty. The first article, from Professor James Bonner and his colleagues at California Institute of Technology, leads off with an important reinterpretation of histones and other chromosomal proteins in light of the complete amino acid sequences recently published for histones II and IV. Bonner's group has long been a pacesetter for other laboratories in this field, by designing and executing experiments unparalleled in their combination of daring, incisiveness, and impeccable biochemical expertise. The subject itself is widely recognized as a key frontier area, which in due course may reveal the secrets of gene regulation.

Certainly no less exciting is the contribution from Professor Bengt Kihlman, who writes about "Molecular Mechanisms of Chromosome Breakage and Rejoining." Dr. Kihlman is director of the Department of Genetics at the University of Uppsala, Sweden, and for some years his book, *The Actions of Chemicals on Dividing Cells*, has been a core reference in this field. His article for this volume goes far toward introducing coherence and logic to a sprawling subject, which in the past has embraced a whole menagerie of radiation, biochemical, and autoradiographic studies done at the light microscope level with a wide variety of plant, animal, and human species. It is amazing that this is the first time an expert in any field dealing with chromosome anomalies has attempted to relate that science to the ultrastructure of chromosomes, as known from electron microscope studies.

Fully as significant as the preceding is a new article by Professor

Andrew Bajer and his close colleague, Dr. Molè-Bajer, who continue the unique collaboration that has given the world both a vast amount of information about the mitotic spindle, and two beautiful little girls. Those familiar with the history of this field know that the Bajers have continually set higher standards of excellence by their skillful use of time-lapse cinemicrography combined with Nomarski phase contrast instruments, and more recently by the use of the electron microscope to examine individual cells filmed during normal mitosis up to the moment of fixation. In this way they have approached more closely than any other investigators toward the study of *living* cells by electron microscopy.

Turning attention from mitosis to meiosis, we are fortunate in having an important new article about synaptonemal complexes from the laboratory of Dr. J. Roberto Sotelo and his colleague, Rodolfo Wettstein. As director of the Department of Cell Ultrastructure at the Institute for Investigation in the Biological Sciences, Montevideo, Uruguay, Dr. Sotelo has directed research that is famous throughout the world for its painstaking excellence. It is thanks to this laboratory alone that we know: every pair of homologous chromosomes has its own synaptonemal complex (SC); every SC is attached at either end to the nuclear envelope; the trajectories of the SC's vary from nucleus to nucleus; supernumerary SC's occur independently of paired homologs; and the fine structure of the SC's changes characteristically from species to species. In this volume, Sotelo and Wettstein take a new look at the structure and function of synaptonemal complexes at a time when many of their earlier inferences have been dramatically confirmed by the first electron micrographs showing intact SC's prepared as whole mounts.

Still another beautiful contribution comes from the laboratory of Professor R. D. Allen, Chairman of the Department of Biological Sciences at the State University of New York in Albany. Long a student of amoebae, and father of the presently accepted fountain zone model of amoeboid movement, Dr. Allen and his collaborator, Dr. McGee-Russell, report new methods for reversibly fixing the structure of unusual pseudopods called filopodia. This variant of amoeboid movement has been difficult to fit into a general concept of protoplasmic flow; however, the innovations which they now report, dramatically clarify the ultrastructure of filopodia and help to settle a long debate about the importance of microtubules in filopod formation.

The application of fundamental research to clinical medicine is very much a part of the *Advances in Cell and Molecular Biology*. In this volume, we are pleased to have an article by Dr. Fritz Lampert, Chairman of Hematology at the University of Munich's Children's Hospital.

Dr. Lampert is a talented physician, dedicated to eradicating the primary life-threatening disease of children, acute leukemia. His approach has been the use of quantitative electron microscopy to detect alterations in the dry mass and structure of chromosomes from malignant cells. Here he reports impressive progress with this exciting method.

The scientist who originated quantitative electron microscopy is Dr. Gunther Bahr, Chief of Biophysics at the Armed Forces Institute of Pathology in Washington, D.C. Dr. Bahr has also provided an important article in this volume, in which for the first time he presents evidence correlating the DNA content of mitochondria with their dry mass. This is particularly significant because mitochondrial DNA occurs in the form of circular molecules having a very uniform size. The suggestion is that a mitochondrion containing only one such molecule must have a minimum dry mass, corresponding to a "unit mitochondrion."

To all of the authors who contributed to this first volume of the *Advances in Cell and Molecular Biology*, thanks are due for highly successful labor and delivery. Credit for easing their pains goes to the many people within Academic Press who attended the birth of this new serial. As for the *Advances*' paternity, responsibility is sure to fall on the shoulders and other parts of the Editor, who can hardly deny cross-antigenicity with his prior offspring, called just plain *Cell and Molecular Biology*. All of us hope that our *Advances* will find good friends everywhere, and that in its journeys to distant parts of the earth, it will bring insight and new knowledge to everyone who values them.

April, 1971

E. J. DuPraw
Stanford, California

THE BIOLOGY AND CHEMISTRY OF CHROMOSOMAL PROTEINS*

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Recent attempts to dissect the chromosomes of eukaryotes and to study their constituent molecules have led to several achievements which have made "histones respectable again" (*Nature* 223:892, 1969). However, serious studies on nonhistone chromosomal proteins are at an early stage. We propose here to discuss both types, but to emphasize those recent studies which have clearly increased our understanding of these proteins and their functions. We will discuss only a minority of the one thousand plus papers that have appeared on these subjects in the last ten years. For other reviews of the recent literature the reader is referred to Stellwagen and Cole (1969b) and to Hearst and Botchan (1970).

I. Characterization of Histones

A. Characteristics and Heterogeneity

There are three classes of histones: lysine-rich, slightly lysine-rich, and arginine-rich. Each class consists of two or more protein species, for which several different systems of nomenclature have been devised. The relationships between these systems and the general characteristics of each protein are presented in Table I. Their amino acid compositions are presented in Table II. Unless otherwise noted, discussions in this article will center around the histones of calf thymus.

Diversity of lysine-rich histones has been studied extensively in several tissues and organisms. Kinkade (1969) demonstrated qualitative differences in the lysine-rich histones of calf, cat, rat, and chicken, but he found only quantitative differences among various tissues, including liver, kidney, spleen, thymus, and erythrocytes. The number of components varied: three in calf spleen, four in cat spleen, and five in rat spleen. Of particular interest was the finding that one of the histone I components in rat contains methionine, an amino acid which is rare in histones; this is a clear example of species specificity at the level of primary structure. Bustin and Cole (1969a) separated histone I from rabbit thymus into nine fractions, eight of which seem to be homo-

TABLE I

PRINCIPAL COMPONENTS OF CALF THYMUS HISTONE AND THE TWO COMMONLY USED SYSTEMS FOR THEIR NOMENCLATURE

Class	Subclass; nomenclature of		Lys/Arg ratio	Molecular weight	Moles/100 moles total histone[c]	N-terminal[d]	C-terminal[d]
	Rasmussen *et al.*[a]	Johns and Butler[b]					
Lysine-rich	Ia	f1	22[e]	21,000[e]	6.7	Blocked	Lysine
	Ib	f1	22[e]	21,000[e]	6.6	Blocked	Lysine
Slightly lysine-rich	IIb1	f2a2	~2.5[f]	13,000–15,000[g]	20.0	Blocked	Lysine
	IIb2	f2b	2.5[h]	13,774[h]	24.6	Proline	Lysine
Arginine-rich	III	f3	0.8[f]	13,000–15,000[g]	18.3	Alanine	Alanine
	IV	f2a1	0.7[i]	11,282[i]	23.8	Acetylserine	Glycine

[a] Rasmussen *et al.* (1962).
[b] Johns and Butler (1962).
[c] Data of Panyim and Chalkley (1969a).
[d] Fambrough and Bonner (1969); Phillips and Simson (1969).
[e] Bustin *et al.* (1969).
[f] Fambrough and Bonner (1966).
[g] Values estimated by comparison with electrophoretic and chromatographic characteristics of histone IIb2.
[h] Iwai *et al.* (1969).
[i] Smith *et al.* (1970).

geneous. Although histone I is more susceptible than other histones to degradation, the authors showed conclusively that these fractions were not degradation products because they all possessed the lysyl-lysine C-terminal and blocked N-terminal residues which characterize histone I. Nevertheless, the differences among the proteins were small; they

TABLE II
AMINO ACID COMPOSITIONS[a] OF HISTONES

Amino acid	Histone I[b]	Histone IIb2[c]	Histone III[d]	Histone IV[e]
Lys	61	20	12	11
Arg	3	8	18	14
His	0	3	2	2
Asp	5	6	6	3
Glu	8	10	15	4
Ser	12	13(14)	5	2
Thr	8	8	10	7
Asn	—	—	—	2
Gln	—	—	—	2
Ala	56	13	18	7
Val	9	9	5	9
Ilu	2	6	6	6
Leu	9	6	12	8
Met	0	1(2)	0	1
Phe	1	2	4	2
Tyr	1	4(5)	2	4
Try	0	0	0	0
Pro	22	6	5(6)	1
Gly	15	7	8	17
Cys	0	0	2	0
Total	212	122(125)	130(131)	102

[a] Compositions are in moles of amino acid per mole of protein.
[b] Bustin *et al.* (1969) for rabbit thymus.
[c] Iwai *et al.* (1969) for calf thymus.
[d] Calculated from data of Fambrough and Bonner (1968b) for calf thymus.
[e] DeLange *et al.* (1969a) for calf thymus.

all had essentially the same size, charge, and amino acid composition. Consequently the heterogeneity may have resulted from differential methylation, acetylation, or phosphorylation (see Section IV). Phosphorylation at a single point in a histone can produce a very altered chromatographic profile on CG-50 Amberlite (Marushige *et al.*, 1969). Therefore, determination of amino acid sequences is generally necessary to show conclusive species specificity in histone primary structure.

The characteristics of the histones II (slightly lysine-rich) are less well determined than those of the other histones, due to difficulties in obtaining pure proteins. However, it is known that there are two subclasses, histone IIb1, and IIb2, and their properties are listed in Table I. The amino acid sequence of histone IIb2 has been determined (Iwai *et al.*, 1969) and will be discussed below. It is important to note that histone f2a1 in the nomenclature of Johns is not a slightly lysine-rich histone, but is of the arginine-rich class, and is referred to in this review as histone IV.

The heterogeneity of histone III, an arginine-rich histone, has been the subject of much controversy. Early experiments (Hnilica and Bess, 1965; Mauritzen *et al.*, 1967; Johns, 1968a) suggested that histone III might consist of as many as 12 components, separable by polyacrylamide gel electrophoresis and other analytical methods based on molecular weight. However, views have changed since the discovery that histone III contains cysteine, an amino acid previously thought not to be present in histones (Blazek and Bukaresti, 1964; Phillips, 1965; Ord and Stocken, 1966c; Jellum, 1966; Sadgopal, 1968; Fambrough and Bonner, 1968b). Calf thymus histone III contains two moles of cysteine per mole of protein and possesses the ability to form polymers of various molecular weights by oxidation of the sulfhydryl groups to form intermolecular disulfides (see Fig. 1). It has been shown by Fambrough and Bonner (1968b) and by Phillips (1967) that the supposed heterogeneity of histone III can be eliminated if pure preparations are reduced with mercaptoethanol prior to gel electrophoresis. Although histone III contains two cysteine residues in calf and HeLa cells (Sadgopal, 1968), in plants it generally contains only one and can form only dimers (Smith *et al.*, 1970; see Fig. 1). Polymerization of histone III seems to occur only in relatively purified preparations; under other conditions, incubation of whole calf thymus histone in a denaturant such as urea results in the disappearance of sulfhydryl groups without formation of polymers, suggesting that intramolecular disulfide bonds have formed (Froehner, 1969). The biological significance of the oxidation state of histone III sulfhydryl groups has been considered by Hilton and Stocken (1966) and by Ord and Stocken (1966a, 1968a,b, 1969). Sadgopal and Bonner (1970b) found that in interphase chromatin these sulfhydryls occur mainly in the reduced form, while in metaphase chromosomes they are oxidized, and histone III is either polymerized or complexed with acid-soluble nonhistone proteins. A very sensitive colorimetric assay for estimating histone thiol directly in acid, recently developed from Saville's method (nitrous acid reaction), should be of use in further studies (Todd and Gronow, 1969).

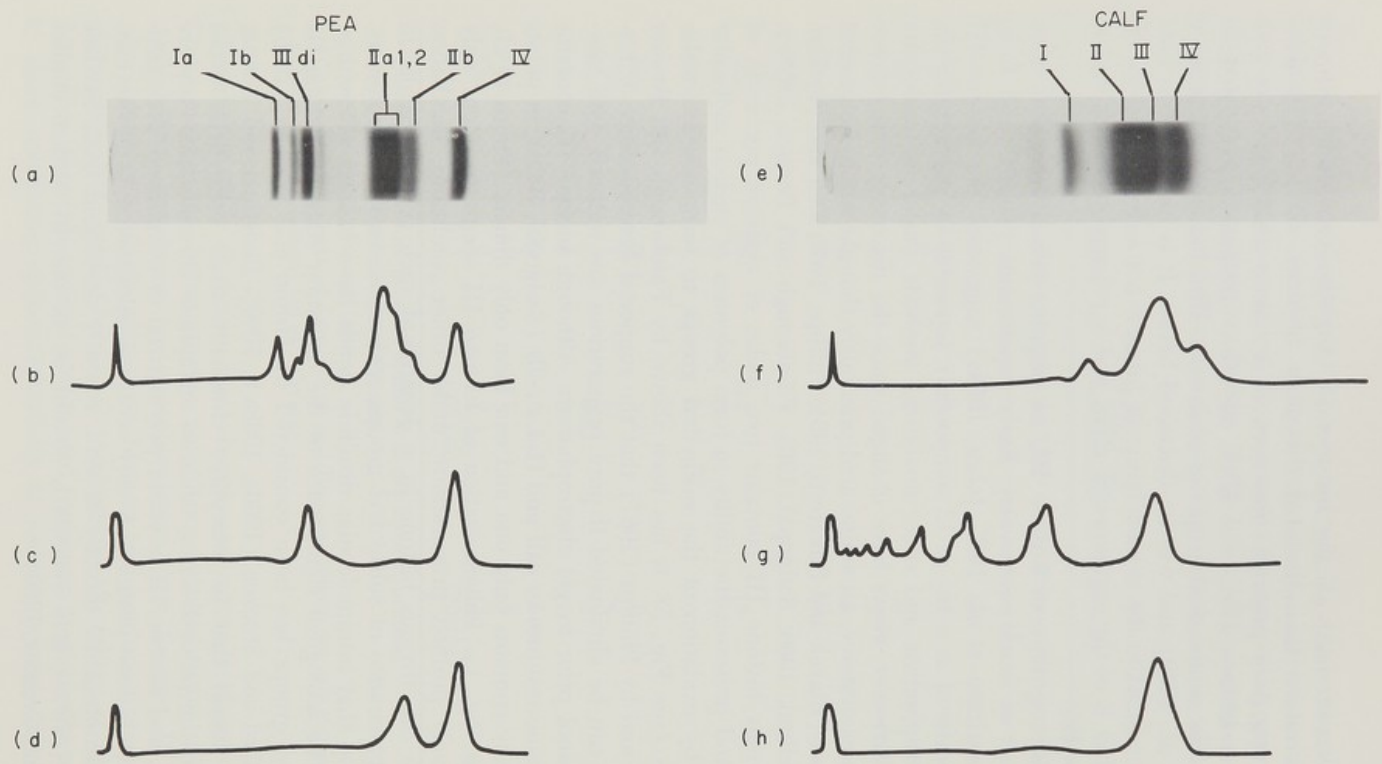


FIG. 1. (a) Polyacrylamide-urea gel of pea whole histone. (b) Densitometer tracing of pea whole histone gel. (c) Pea histone IV + III (dimer). (d) Pea histone IV + III (monomer). (e) Polyacrylamide-urea gel of calf thymus whole histone. (f) Densitometer tracing of calf thymus whole histone gel. (g) Calf thymus histone III (polymers). (h) Calf thymus histone III (monomer).

Histone IV, the second of the arginine-rich histones, has been shown to be a single polypeptide chain. Although it tends to aggregate with histone III during most isolation procedures, it has been purified and its amino acid sequence determined (see below); it contains one methionine. Due to its extremely high arginine content, histone IV is the most basic of the histones, with a calculated isoelectric point near pH 12.

The fact that there are a small number of histone molecules of different primary structure, once a point of contention, has now been generally accepted.

B. Principal Methods of Isolation

An exhaustive review of the procedures for histone isolation and purification has been provided by Butler *et al.* (1968).

Histones occur in chromosomes complexed with DNA in a weight ratio of about 1:1, and can be isolated from purified chromatin by acid extraction (Bonner *et al.*, 1968a). Because extraction with hydrochloric acid produces histone chlorides which are not totally precipitable from acid ethanol solution, are hygroscopic, and are more difficult to redissolve than histone sulfates, 0.4 *N* sulfuric acid is the preferred reagent (Fambrough, 1968). Acid extraction of whole nuclei or even of whole cells has been used (Cruft and Leaver, 1961; Bellair and Mauritzen, 1967), but this procedure results in contamination of the histones by other basic proteins.

The most widely used method for purification of the histone fractions is chromatography on IRC-50 Amberlite resin using a guanidine hydrochloride gradient (Luck *et al.*, 1958; Rasmussen *et al.*, 1962). The three classes are eluted in their order of increasing arginine content, lysine-rich (I), slightly lysine-rich (II), and arginine-rich (III, IV). Each class is heterogeneous and slightly cross-contaminated by other classes, although less so than when prepared by other methods. Rechromatography using a shallower gradient resolves lysine-rich histone into several polypeptides (Bustin and Cole, 1969a). Attempts to fractionate histones by exclusion chromatography have met with only limited success (Cruft, 1961; Bellair and Mauritzen, 1964), except for the isolation of histone IV (Fambrough and Bonner, 1969).

For analytical purposes, disc electrophoresis in pH 4.3 urea polyacrylamide gels has proved useful, and it requires only small amounts of material (Bonner *et al.*, 1968a). Pictures and densitometric tracings of typical gels from whole histone of calf thymus and pea bud are shown in Fig. 1. Modifications of this technique have been used to

demonstrate microheterogeneity of histones (Kinkade, 1969; Panyim and Chalkley, 1969a). Preparative disc electrophoresis successfully separates histone III and IV (Fambrough and Bonner, 1969).

A crude fractionation of histone classes can be achieved by selective extraction of chromatin with acid and acidified ethanol, followed by selective precipitation with ethanol and acetone (Johns and Butler, 1962; Johns, 1964, 1967, 1968b). However, this method yields fractions, each of which is appreciably contaminated with others, and it often does not work well for tissues other than calf thymus. Chromatography on carboxymethyl cellulose yields somewhat purer fractions (Phillips and Johns, 1959).

Histone I can be prepared by pulverizing tissue in solid carbon dioxide and extracting with 3.5% trichloroacetic acid (Bustin and Cole, 1968b). This fairly pure preparation of lysine-rich histones can then be separated into several polypeptides by other methods discussed previously.

C. Occurrence of Unusual Amino Acids

The histones have been found to contain several unusual amino acids. DeLange *et al.* (1968a) detected the presence of ϵ-*N*-acetyllysine in histone IV of calf thymus. Murray (1964) showed that histone IIa from calf thymus contains 0.3 to 0.5 mole % of ϵ-*N*-methyllysine, while histones III and IV contain slightly more. ϵ-*N*-Dimethyllysine has been detected in the arginine-rich histones of calf thymus (Paik and Kim, 1967a), and the trimethyl derivative has also been reported (Hempel *et al.*, 1968). In addition to lysine derivatives, Paik and Kim (1969b) reported the detection of two derivatives of arginine in calf thymus histone: ω-*N*-monomethylarginine and α-*N*-methyl, guanidino-methylated arginine. Another derivative of a basic amino acid, 3-methylhistidine, has been isolated by Gershey *et al.* (1969). Finally, many of the histones have N-terminals blocked by acetate (Fambrough, 1968; Phillips, 1968). It is noteworthy that these various alterations make the proteins less basic.

Although there have been reports that serine-*O*-sulfate and threonine-*O*-sulfate occur in histones, these have been shown to be artifacts due to the hydrolysis of histone sulfates with HCl prior to amino acid analysis (Murray and Milstein, 1967). Such artifactual esters could also lead to low values for serine and threonine contents. On the other hand, the phosphate esters of serine in histones are not artifactual and will be discussed in Section IV.

D. Unusual Histones

A unique minor histone occurs in the nucleated erythrocytes of birds, fish, and frogs (Edwards and Hnilica, 1968; Neelin, 1968). This histone is of the slightly lysine-rich class (a lysine:arginine ratio of about 2), but it also contains a large amount of serine (approximately 12%) and an extraordinarily high content of basic amino acids. Although it has been suggested that this histone is involved in maintaining the highly repressed state of the erythrocyte chromatin, direct evidence is lacking.

The protamines, small basic proteins found in bird and fish sperm, are usually considered as a separate class of proteins; in this article, protamines will be discussed only in the sections on protein evolution and phosphorylation. A recent review by Stellwagen and Cole (1969b) is recommended as a source for more information about protamines. Other sperm histones have been reviewed by Bloch (1969).

E. Primary Structure of Histones

Recent work on the primary structure of the histones has revealed that the basic amino acids are not distributed randomly throughout the molecule, but are clustered at one end. For example, Bustin *et al.* (1969) determined the distribution of lysine residues in a histone I from rabbit thymus. The molecule was divided into two approximately equal parts, as shown in Table III; it could then be shown that the half corresponding to the carboxyl end (116 residues) contained 45 of the total 68 lysine residues, suggesting that this end of the molecule may be designed to interact strongly with DNA in the manner of polylysine. This idea was further supported by the fact that 16 of the 22 prolines were also located in the same half, tending to force the carboxy-terminal half of the polypeptide into an extended random coil configuration. By contrast, the amino terminal half of the protein contained 100 amino acids, including 13 of the total 16 acidic residues and 16 of the total 22 hydrophobic residues. In general this N-terminal half of histone I resembles small nonhistone proteins. The sequence of the first 74 N-terminal amino acids of a rabbit thymus histone I has been determined, and as expected there is no sequence homology with histones IV or IIb (Rall and Cole, 1970).

Histone IIb2 of calf thymus, which has recently been sequenced by Iwai *et al.* (1969), also shows a remarkably nonrandom distribution of lysine and arginine residues (see Fig. 2). The N-terminal region of the molecule, comprising residues 1 to 56, is especially rich in lysine

and possesses several clusters of up to four consecutive basic amino acids. By contrast the C-terminal region, comprising residues 71 to 125, is poor in lysine but relatively enriched in arginine. The middle region, comprising residues 57 to 70, has the longest spacing between basic residues. In this region there are 14 nonbasic residues, including all of the methionine and phenylalanine, as well as half the aspartic and asparagine residues. Other amino acids are also irregularly distributed. For example, four of the six total proline residues are included among the 11 N-terminal amino acids, while three of the five total tyrosine residues are included in the sequence between lysine 33 and lysine

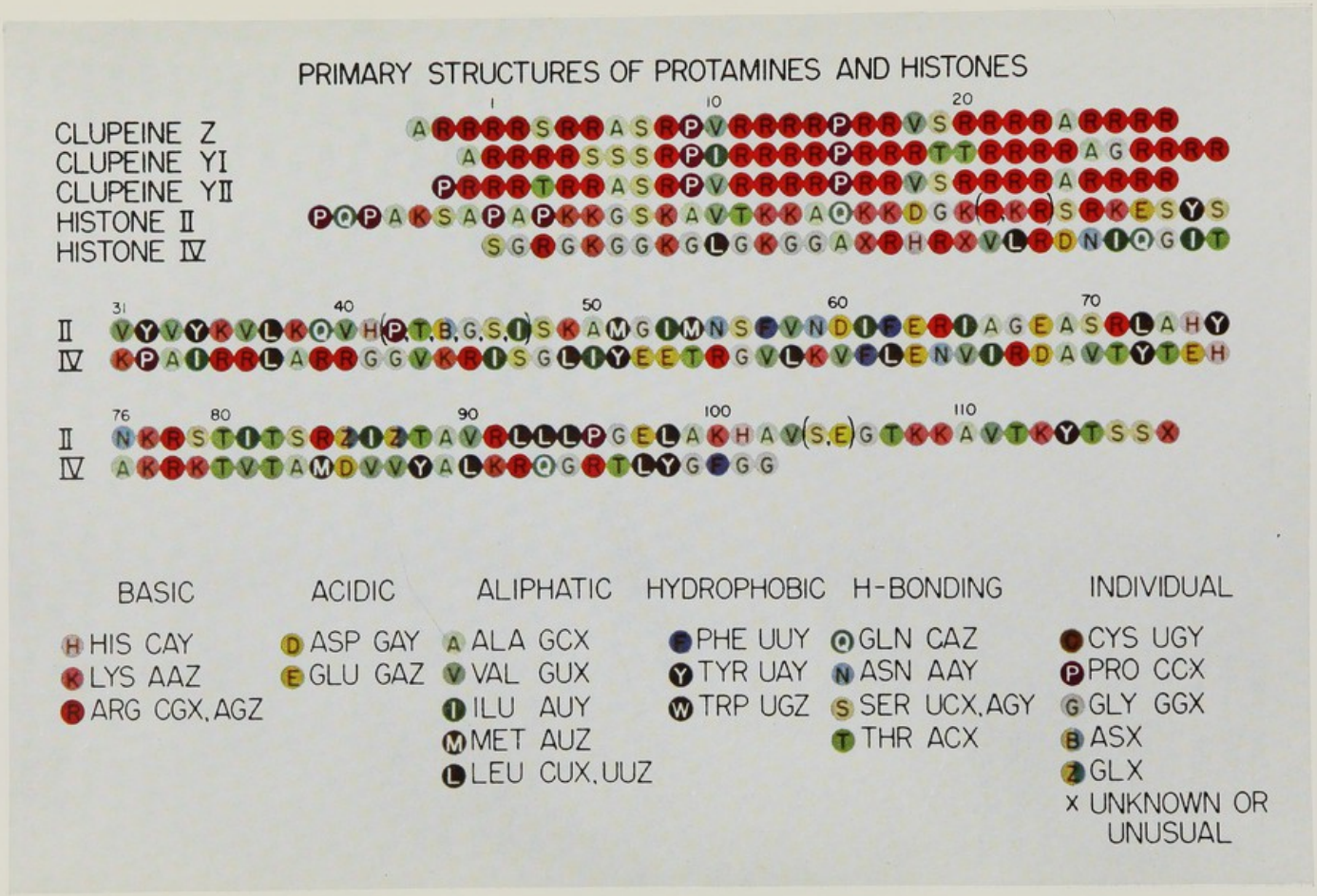
TABLE III
DISTRIBUTION OF AMINO ACIDS IN PEPTIDES OF LYSINE-RICH HISTONE Ib (RABBIT THYMUS)[a]

Residues	N-terminal part	C-terminal part
Total	100	116
Lysine	16	45
Proline	6	16
Acidic	13	3
Hydrophobic	16	6

[a] Adapted from Bustin *et al.* (1969); Bustin and Cole (1969b).

42. In general the C-terminal region has more hydrophobic residues, as well as more hydroxyamino acids, especially threonine. An extremely interesting section of the molecule is the C-terminal end, in which the last five residues all contain hydroxyl groups, suggesting that they might participate in β-structure with other proteins. It appears that portions of the N- and C-terminal regions are designed for binding to DNA, while the middle region may interact with other factors.

The primary structure of histone IV, containing 102 amino acids, is almost identical in calf thymus (DeLange *et al.*, 1969a; Ogawa *et al.*, 1969) and in pea seedlings (DeLange *et al.*, 1969b; see Figure 2). Again, clustering of basic residues is common. The N-terminal region (residues 1–45) contains 63% of the basic amino acids of the total molecule, including one polypeptide of five consecutive basic residues. It is noteworthy that lysine 16 is acetylated to the extent of 50% both in calf thymus and in pea. Only one of the hydroxyamino acids and one of the acidic residues is located in the N-terminal region. By contrast, the C-terminal end (residues 46–102) contains approximately two thirds of the hydrophobic residues, including all six of the aromatics. Due to its high con-


PRIMARY STRUCTURES OF PROTAMINES AND HISTONES
CLUPEINE Z
CLUPEINE YI
CLUPEINE YII
HISTONE II
HISTONE IV
II
IV
II
IV
BASIC
HIS CAY
LYS AAZ
ARG CGX, AGZ
ACIDIC
ASP GAY
GLU GAZ
ALIPHATIC
ALA GCX
VAL GUX
ILU AUY
MET AUZ
LEU CUX, UUZ
HYDROPHOBIC
PHE UUY
TYR UAY
TRP UGZ
H-BONDING
GLN CAZ
ASN AAY
SER UCX, AGY
THR ACX
INDIVIDUAL
CYS UGY
PRO CCX
GLY GGX
ASX
GLX
x UNKNOWN OR UNUSUAL

tent of positive charges, it seems likely that the N-terminal end of histone IV may be the primary DNA binding site. The C-terminal region, in view of its richness in hydrophobic residues and in the hydrogen-bonding hydroxyl groups of threonine and serine, may have a specific conformation not dictated by interaction with nucleic acids (DeLange *et al.*, 1968b).

It seems quite probable then that histones contain specific regions for interaction with nucleic acids, and that for histones II and IV this is the N-terminal end. Supporting this idea is the fact that protamines, which bind very strongly to DNA, exhibit a high degree of functional homology with the N-terminal end of the histones (see Fig. 2). Other regions of the histone may be reserved for a different function, possibly interactions with other histones or other proteins of the chromosome. Although further information is needed to determine if this hypothesis is correct, the determination of sequences for other histones should shed more light on this proposal.

II. Occurrence of Histones

A. Basic Proteins of Bacteria

In order to learn something of the function of histones, several groups have investigated whether prokaryotes as well as eukaryotes contain histones. In a study of the properties of *E. coli* deoxyribonucleoprotein (DNP), Raaf and Bonner (1968) isolated an 85 S DNP with a protein:DNA ratio of 2.3. These bacterial proteins were less basic than histones, did not stabilize DNA against thermal denaturation, and possessed an amino acid composition similar to that of whole bacterial protein. The isolation of "histones" from *Staphylococcus aureus* has been reported (Cruft and Leaver, 1961), but inasmuch as these were isolated from whole cells instead of chromatin it is probable that they are ribosomal in origin. In a later study of various types of bacteria (Leaver and Cruft, 1966), it was found that whole bacteria contain 0.1 to 1.0% basic protein, but that most of this is ribosomal protein. Present evidence

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Fig. 2. The one-letter codes for the amino acids are standard IUPAC nomenclature. Sequences have been aligned to show maximum homology. Residue 118 in histone II is hydroxylysine. In histone IV, residue 1 is acetylserine, residue 16 is ϵ-*N*-acetyllysine (50% acetylated), and residue 20 is methyllysine. In pea histone IV, residue 60 is isoleucine and residue 77 is arginine. *Note added in proof:* in the final published sequence of histone II, the Lys at 109 is omitted and a Lys inserted between 21 and 22 (our numbering) making the sequence 21–26 Lys-Lys-Arg-Lys-Arg-Ser; the sequence 42–47 is Pro-Asp-Thr-Gly-Ile-Ser; Glx at 85 is Glu and Glx at 87 is Gln; 104–105 is Ser-Glu (Iwai *et al.*, 1970).

suggests that bacteria may contain proteins associated with their DNA, but these proteins are not as basic as histones and their exact function is presently unknown.

B. Histones of Eukaryotes: Structural and Evolutionary Relationships

Histones are present in most unicellular as well as multicellular organisms which have a defined nucleus and contain chromosomes that undergo condensation. For example, histones with an electrophoretic pattern similar to those of calf thymus have been extracted from the myxomycete *Physarum polycephalum* (Mohberg and Rusch, 1969). The macronuclear chromatin of *Tetrahymena pyriformis* also contains histones which are similar to those of calf thymus as judged by gel electrophoresis (Griffith and Bonner, 1970a). Dwivedi *et al.* (1969) reported that the chromatin in *Neurospora crassa* does not contain histones, and the same is true of the chromatin of *Microsporum gypseum*, of the Fungi Imperfecti (Leighton *et al.*, 1970). It is important to note that chromatography on CG-50 is a necessary step in histone isolation from lower organisms (Luck *et al.*, 1958). Omission of this procedure may result in contamination with ribosomal proteins, thus preventing the detection of histones by the normal procedures. Such contamination may very well explain previous reports of the absence of histones in some lower organisms.

With regard to plants and animals, histones have been isolated and characterized from many different species. Two general conclusions can be drawn: First, it is evident that all higher organisms investigated possess the same general distribution of histones. The pattern of histones has been shown to be very similar in a clam (Cozolluela and Subirana, 1968), trout (Palau and Butler, 1966), *Drosophila* (Griffith, 1969; Dick and Johns, 1969c), rat thymus and spleen, leukemic rat liver and spleen (Hnilica *et al.*, 1962), rat tumors induced by three different viruses (Boulanger *et al.*, 1969), calf thymus, liver, and spleen (Hnilica *et al.*, 1962), chick liver and spleen (Neelin and Butler, 1961), *Vicia faba*, *Allium cepa*, and *Pisum sativum* (Cohn and Quatrano, 1965), and various human tissues (MacGillivray, 1968). According to Cole and co-workers there is some variation in the lysine-rich histones (Bustin and Cole, 1969a); although much of this may be due to microheterogeneity (Sections V,D and V,E), there is some evidence for differences in primary structure (Kinkade, 1969). Furthermore, though no higher organism has yet been found which does not have some of each class of histones, quantitative differences definitely do exist.

Second, the primary structure of the histone fractions, particularly the arginine-rich histones, has been highly conserved throughout evolu-

tion. For example, histone IV has been shown to have a very similar amino acid sequence in peas and cows. Only two very conservative substitutions were found, i.e., one valine in calf is replaced by isoleucine in pea, and one lysine in calf is replaced by arginine in pea (DeLange *et al.*, 1969a,b). Desai *et al.* (1969) sequenced the C-terminal end of histone IV from bovine lymphosarcoma, Novikoff hepatoma, and fetal calf thymus, finding no differences from the sequence in peas. Fambrough and Bonner (1968b) studied the primary sequence of histone III from pea and calf by peptide mapping; 26 of 29 peptides in histone III are common to both pea and calf. Finally, studies by high resolution gel electrophoresis also indicate charge invariance for histone III from a wide variety of plants and animals (Chalkley, 1969). Similar studies on other histone fractions have shown less, although still considerable homology (Hnilica *et al.*, 1963; Hnilica, 1966; Fambrough, 1968).

The invariance of the primary structure of histone IV from two phylogenetically distant organisms is unique. Less than 2% of the residues are altered in histone IV from peas and cows; by contrast, the sequences of cytochrome *c* from wheat (Stevens *et al.*, 1967) and from cow (Yasunobu *et al.*, 1963) differ in over 30% of the amino acid residues. It is probable that all but a few very conservative mutations in the histone IV gene are lethal, suggesting that each residue must have a critical function. If this is true, then histone IV must be part of a highly conserved complex. The most obvious partner in such a complex, the tertiary structure of which has undergone no major change during evolution, is the sugar-phosphate backbone of the DNA double helix. Possibly some or all of the other histones may have been highly conserved as well, and they too may participate in complex formation with histone IV. Finally, nonhistone chromosomal proteins, particularly those involved in meiosis and mitosis, would be expected to interact with histones, and these may also have been conserved. Whatever the cause, no other protein yet studied has been conserved to the remarkable extent of histone IV.

C. Comparison of Primary Structures of Histones and Protamines

The amino acid sequences of two histones and three protamines, aligned so as to exhibit maximum homology, are shown in color-coded form in Fig. 3. This color code illustrates not only functional homology, in the sense of similar amino acid side chains, but also the rate of mutation of one amino acid to another (i.e., amino acids of the same general color tend to interchange with each other at higher rates than those of different colors). For a comprehensive discussion of these mutation rates the reader is referred to Dayhoff and Eck (1968).

An interesting aspect of the histone sequences is the regular spacing of basic residues in the N-terminal half. The spacing of basic amino acids, frequently at every fifth position, may be necessary for binding of the molecules to DNA. Occurrence of two adjacent basic residues in also frequent.

Protamine sequences show remarkable homology, particularly with respect to arginine and proline (Ando *et al.*, 1962; Ando and Suzuki, 1966, 1967). The appearance of the pentapeptide X-Arg-Arg-Arg-Arg, in which X is an aliphatic amino acid, is prevalent throughout these molecules, including salmine and iridine (Ando and Watanabe, 1969). Black and Dixon (1967) have proposed that the protamines evolved through a series of partial gene duplications of the ancestral pentapeptide Ala-Arg-Arg-Arg-Arg, followed by a small number of single nucleotide mutations. On the other hand, it appears that histones evolved before protamines, inasmuch as the former are found in all eukaryotes and in organisms lower than those which possess protamines. There is a possibility that protamines may have evolved from the basic end of a histone molecule. Comparison of the protamine and histone sequences presently available indicate some homology between the two; however, evolution of clupeine YII from the N-terminal region of histone II would require eleven single base changes and eight double base changes. Comparisons of the remaining protamines with both histones II and IV reveal even less homology. Consequently we regard it as unlikely that protamines evolved from histones and suggest that the sequence homology noted is probably due to constraints imposed upon the two kinds of molecule by their similar function.

Likewise, there seems to be only functional sequence homology between histones II and IV. DeLange *et al.* (1969b) noted that there are several similar regions in histone IV which require only single base changes to make them equivalent; this molecule may therefore have arisen from a few ancestral peptides by gene duplication and point mutations in a manner analogous to that proposed by Black and Dixon (1967) for the protamines. However, not enough sequences are available to deduce the evolutionary history of the histones.

III. Interaction of Histones with DNA

A. Introduction

The roles of the individual histones in the structure and function of deoxyribonucleoproteins have been studied in two principal ways:

(a) investigation of the properties of nucleohistones reassociated from DNA and individual histones (Huang *et al.*, 1964; Johns and Butler, 1964; Olins, 1969; Shih and Bonner, 1970b); and (b) investigation of the properties of native chromatin after selective removal of individual histones (Murray and Peacocke, 1962; Ohba, 1966a,b; Ohlenbusch *et al.*, 1967; Murray, 1969; Levinson *et al.*, 1970; Smart and Bonner, 1970b,c). The first part of this section concerns the biochemical and biophysical properties of DNA reassociated with individual histones and with other native or synthetic basic polypeptides. The second part will deal with the biochemical and biophysical properties of native chromatin which has been partially dehistonized by sodium deoxycholate or by ionic dissociating agents such as sodium chloride, sulfuric acid, sodium perchlorate, or guanidine hydrochloride.

Increasing concentrations of ionic dissociating agents selectively remove histone I (lysine-rich) at lower concentrations, while higher concentrations remove histone II (slightly lysine-rich) and histones III and IV (arginine-rich; Georgiev *et al.*, 1966; Ohlenbusch *et al.*, 1967; Fambrough and Bonner, 1968a; Murray, 1969; Tuan and Bonner, 1969; Levinson *et al.*, 1970; Smart and Bonner, 1970b). On the other hand, increasing concentrations of sodium deoxycholate (DOC) dissociate histone II at lower concentrations, while slightly higher concentrations remove histones III and IV. Histone I is the last histone fraction to be removed by DOC (Smart and Bonner, 1970a). Comparison of the results obtained by the use of different methods for selective removal of histones from native chromatin has allowed a much clearer interpretation of the roles of the various histones in the structure and function of chromatin.

B. Reassociation

1. *Methods*

The reassociation of DNA with histone fractions or with other native or synthetic polypeptides is generally accomplished by either (a) direct mixing of the DNA and polypeptide, previously dissolved in the chosen final buffer, or by (b) mixing the DNA and polypeptide in a high ionic strength buffer followed by slow removal of the salt by gradient dialysis. A useful modification of the second method involves gradient dialysis in the presence of 5 *M* urea (Shih and Bonner, 1970a,b).

2. *Structural Modifications of DNA*

Olins (1969) found that DNA–histone I complexes reassociated by salt gradient dialysis, in contrast to pure DNA, exhibit almost complete

renaturation after partial thermal denaturation. This renaturation is observed at protein cation/DNA phosphate (+/−) ratios as low as 0.08, which produce only a very small stabilization against thermal denaturation. Olins (1969) suggested that the rapidity and high yield of renaturation for such complexes implies that histone I molecules act as links between the complementary DNA strands, preventing extensive strand separation and facilitating nucleation. At higher (+/−) ratios, the binding of basic polypeptides to DNA appears to be completely cooperative (Shih and Bonner, 1970a,b).

Changing the ionic environment of a constrained length of DNA is known to cause severe strain and concomitant structural changes in the double-stranded molecule. For example, the degree of supercoiling of covalently closed circular DNA's varies in different ionic environments (Wang *et al.*, 1968). Because the close association of histone positive charges with DNA negative phosphate groups must involve major changes in the local ionic environment of the DNA duplex, we might expect structural changes to be associated with the cooperative binding of histones to DNA.

Using polylysine as the complexing polypeptide, Olins *et al.* (1968) found that for complexes reconstituted by gradient dialysis a sequence of eight or more lysyl residues are required to achieve biphasic melting. They also observed that tetralysine does not preferentially stabilize A-T base pairs, but that polylysine molecules of length greater than eight residues do. Shapiro *et al.* (1969) reported that the structural changes associated with polylysine complexes formed by direct mixing in 1 *M* NaCl required polylysine of greater than seven residues. Likewise, Kawashima *et al.* (1969) found that DNA–ornithine complexes must include a polypeptide longer than six residues to exhibit peptide-dependent structural changes in the DNA. It appears that physicochemical changes in the properties of DNA reassociated with histones or other basic polypeptides are due to more than simple charge neutralization.

It is also true that the method of reassociation has important effects on the physicochemical properties of such complexes. For example, not only are DNA–polylysine complexes 20 to 30 degrees more thermally stable than DNA–histone I complexes (Olins, 1969), but also direct mixing of DNA and polylysine yields complexes of T_m several degrees lower than those of complexes prepared by gradient dialysis (Olins *et al.*, 1968); these in turn are several degrees less stable than those formed by gradient dialysis in the presence of 5 *M* urea (Shih and Bonner, 1970a).

In a given DNA–homopolypeptide complex the T_m of the first transition ($T_{m,1}$) is constant at all (+/−) ratios and identical to that of the

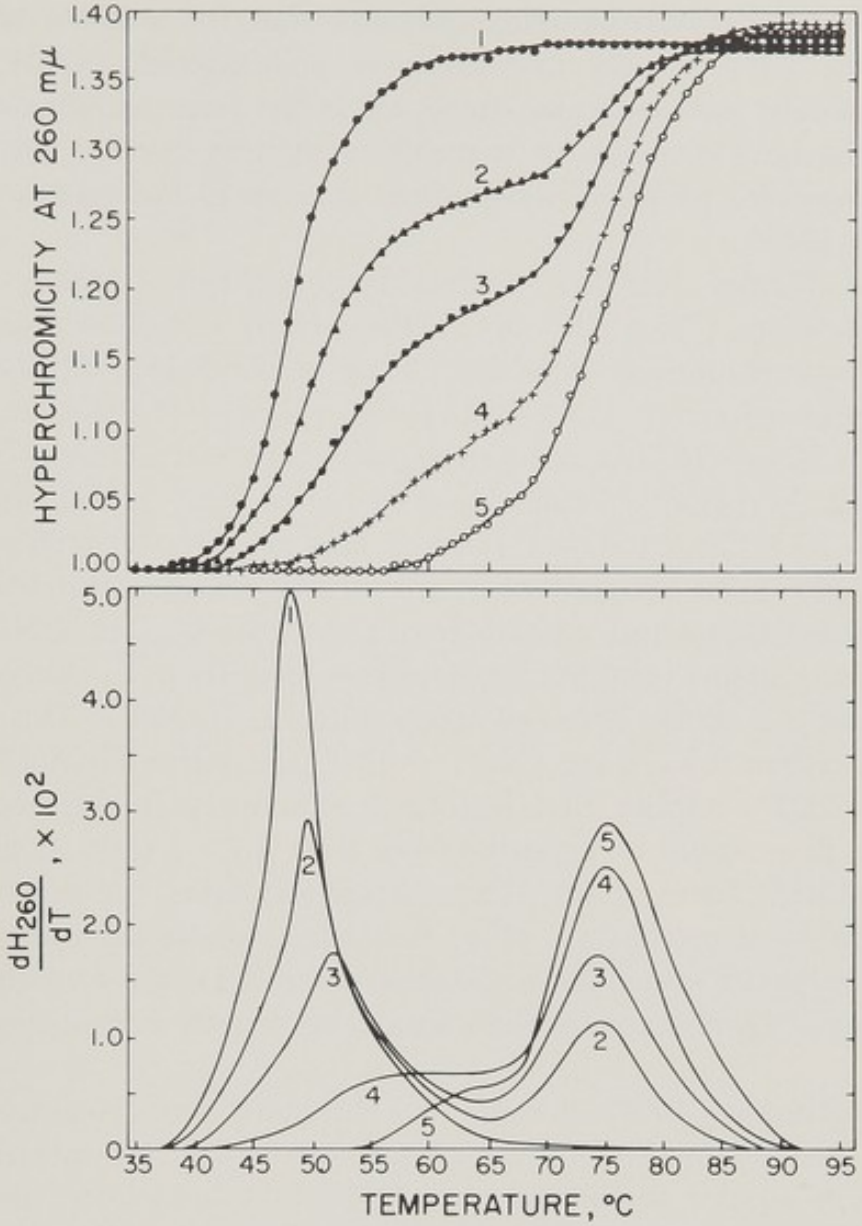

FIG. 3. Melting profiles of DNA–histone Ia complex. Nucleohistone Ia with (lysine + arginine)/nucleotide ratio of 0 (No. 1), 0.20 (No. 2), 0.40 (No. 3), 0.60 (No. 4), and 0.80 (No. 5) were melted in 2.5×10^{-4} *M* of sodium EDTA, pH 8.0. At the top are melting profiles and at the bottom, their derivative curves (Shih, 1969).

pure DNA (Olins *et al.*, 1967, 1968; Matzuo and Tsuboi, 1969; Shih and Bonner, 1970a). In contrast, the $T_{m,1}$ of DNA–protamine (Olins *et al.*, 1968; Shih and Bonner, 1970a) and DNA–histone complexes (Olins, 1969; Shih and Bonner, 1970a) increases with increasing (+/—) ratios. If the reconstitution is done carefully by salt gradient dialysis (especially in the presence of 5 *M* urea) the T_m of the second transition ($T_{m,2}$) remains constant for each DNA–polypeptide complex (Olins *et al.*, 1968; Olins, 1969; Shih and Bonner, 1970a,b). The constancy of $T_{m,2}$ for a

given DNA–polypeptide complex indicates that for a given method of reassociation there is only one kind of well-ordered, highly specific, and energetically well-balanced structure in the fully complexed regions of DNA. Figure 3 presents an example of melting profiles for a DNA–histone Ia complex prepared by gradient dialysis in the presence of 5 *M* urea (Shih, 1969).

Shih and Bonner (1970b) compared the melting profiles of DNA–histone complexes (2.5×10^{-4} *M* EDTA, pH 8) in which about 30% of the total hyperchromicity was due to the second-step transition. This hyperchromic value for DNA–histone Ia ($T_{m,2} = 75.4°C$) and DNA–histone Ib ($T_{m,2} = 76.7°C$) complexes was achieved at a (+/−) ratio of about 0.2. For DNA–histone IIb ($T_{m,2} = 81.5°C$) and DNA–histone IV ($T_{m,2} = 83.7°C$) a (+/−) ratio of approximately 0.4 was required to achieve the same amount of well-ordered complex formation. It is also significant that Olins (1969) found that the $T_{m,2}$ of DNA–histone I complexes is almost completely insensitive to shifts in the ionic strength of the melting medium between 0.005 and 0.1 *M* NaCl. This, coupled with the fact that $d\,T_m/d \log (Na^+) = 18°C$ for native DNA (Dove and Davidson, 1962), implies that histone I effectively shields more DNA phosphates than would be expected from the (+/−) ratio. It seems that histone I shields some of the DNA phosphates from the aqueous ionic environment by a mechanism other than electrostatic neutralization. By contrast, the positive charges of histones II and IV are *less* effective in neutralizing DNA phosphates than would be expected from the (+/−) ratios.

Inoue and Ando (1968) showed that the molecular geometry of DNA contained in a DNA–clupeine complex, as evidenced by optical rotatory dispersion, is similar to that of pure DNA in solution. However, DNA in a DNA–$(Arg)_{20}$ complex is significantly different. Likewise, Shapiro *et al.* (1969) reported that the rotation/residue in the region of 250 to 290 mμ is 50 to 1000 times as large for DNA contained in a DNA–polylysine complex as for pure DNA, indicating a large change in the DNA secondary structure. By contrast, Olins (1969) observed only minimal differences in the absorption spectra and circular dichroism of pure DNA and DNA–histone I complexes. Consequently it appears that the spacing of positively charged groups in polylysine and polyarginine produces severe strain on the DNA duplex, whereas the spacing and flexibility of positively charged groups in the protamines and histone I produces only minimal changes in DNA secondary structure. It should be noted that, according to Inoue and Ando (1968), mixing clupeine with DNA at low ionic strength results in two types of complex: the first forms more rapidly, is less stable, and possesses an Arg/phosphate

ratio of 1.0, while the second forms more slowly, is more stable, and possesses an Arg/phosphate ratio of 0.75.

Lees and von Hippel (1968) developed an interesting membrane filtration method for studying hydrogen exchange kinetics in insoluble DNA–polylysine complexes. The results indicated that about one fourth of the DNA interchain hydrogens are shifted into an "instantaneously" exchanging class on interaction of the DNA with polylysine. In the remaining hydrogens, exchange was essentially unaffected, a result which they regarded as consistent with a conformational change in the DNA. They also reported that the peptide hydrogens exchanged as a single class, with one hydrogen per lysyl. In the DNA–polylysine complex the peptide hydrogens exchanged more slowly, suggesting that these hydrogens were partially shielded from the aqueous environment.

Although studies on the sedimentation and other hydrodynamic properties of reassociated DNA–polypeptide complexes are difficult because of a concentration-dependent aggregation, Olins (1969) found that DNA–histone I complexes at infinite dilution behave like single DNA molecules with attached histones.

3. *Precipitation of DNA–Histone Complexes*

Johns and Forrester (1969) observed that histone I is unable to precipitate DNA from water even at four times excess. In 0.14 *M* NaCl, on the other hand, 0.8 mg of histone I precipitates 1 mg of DNA, while in 0.07 *M* $MgCl_2$ only 0.4 mg of histone I is required for this effect. In general, histone I is about twice as effective in precipitating DNA as are the other histones (Johns and Butler, 1964). This fits well with the amino acid composition data of Fambrough and Bonner (1966), which shows that for histone I the net positive charge per amino acid residue (arginine + lysine) — (glutamic acid + aspartic acid) is approximately twice that of the other histones. Reassociation experiments conducted with an excess of mixed histones show that histones III and IV combine preferentially with DNA; histone II is next in order of preference and histone I last (Johns and Butler, 1964). As will be discussed, the order of removal of the various histones by increasing concentrations of ionic dissociating agents is the reverse of this, i.e. histone I > histone II > III and IV.

4. *Template Activity*

The effects of synthetic and native basic polypeptides on the ability of DNA to act as a template for RNA synthesis in the presence of

E. coli RNA polymerase has been studied extensively (Huang *et al.*, 1964; Hilton and Stocken, 1966; Skalka *et al.*, 1966; Shih and Bonner, 1970a,b; Spelsberg *et al.*, 1969). Shih and Bonner (1970a,b) used very pure histones and other basic polypeptides, as well as an optimum association method (salt gradient dialysis in the presence of 5 *M* urea), providing a uniform basis for comparison of DNA complexed with different basic polypeptides. They found that the template activity of such complexes is proportional to their fractional content of free DNA segments. The complexed DNA regions were quantitatively blocked and did not act as templates. In addition, the authors' kinetic analysis of template behavior revealed two different modes of inhibition by polypeptides. If the template was in a finely dispersed state it was available to the enzyme, as shown by the fact that equal concentrations of complex and of pure DNA produced half-saturation of a given amount of enzyme; under these conditions, inhibition of RNA synthesis was probably due to interference with local unwinding of the DNA duplex. On the other hand, when the template was in a highly aggregated state, it became partially unavailable to the enzyme and the amount of complex required to bind a given amount of RNA polymerase was drastically increased.

Studies in several laboratories have shown that histones are capable of inhibiting various DNA polymerase systems as well (Billen and Hnilica, 1963; Schwimmer and Bonner, 1965; Schwimmer and Aronson, 1967; Wood *et al.*, 1968). Lysine-rich histone (histone I) shows the strongest inhibitory effects for a DNA polymerase reaction (Hnilica and Billen, 1964; Schwimmer, 1965; Wood *et al.*, 1968). In general, there appears to be no nucleotide specificity for inhibition of DNA synthesis by various histone fractions (Schwimmer and Aronson, 1967; Wood *et al.*, 1968). Native chromatin, even if inactive in support of RNA synthesis by RNA polymerase, is active in support of DNA synthesis by DNA polymerase (Schwimmer and Bonner, 1965).

5. *Specificity and Location of Binding*

Leng and Felsenfeld (1966) observed that if DNA and polylysine (degree of polymerization approximately 100) were mixed in 1 *M* NaCl and allowed to reassociate at that ionic strength, the polylysine exhibited an almost perfect selectivity for A-T-rich DNA (as judged by preferential precipitation). They also reported that under these conditions polyarginine interacts about equally with DNA's of base composition in the range 40–60% G-C, but exhibits a slight preference for DNA of composition 72% G-C, and a marked preference for the homopolymer dG + dC. Although these preferential interactions are of interest, it has

not been shown that they play a significant role in the construction of chromosomes. In fact, Leng and Felsenfeld (1966) found that the selective effects were abolished if the reactions were carried out in 0.1 *M* rather than 1.0 *M* NaCl. Although Olins (1969) found that polylysine exhibits a preference for A-T-rich DNA upon gradient dialysis reassociation, no similar specificity was observed when lysine-rich histone I was complexed with DNA under the same conditions. Johns and Butler (1964) also found a lack of specificity in binding of histone I or histone III to DNA.

Molecular model building shows that a polyarginine chain can fit snugly into the small groove of a DNA double helix, with each arginine side chain meeting each DNA phosphate (Wilkins, 1956). Lysine side chains are only slightly shorter than those of arginine and may be able to assume the same structure. In calf thymus histone IV, for which DeLange *et al.* (1969a) have analyzed the complete amino acid sequence, the positively charged residues are grouped into two major and two minor clusters: (*a*) residues 1 to 23; (*b*) 35 to 45; (*c*) 75 to 79; (*d*) 91 to 95 (see Fig. 3).

Model building experiments by Shih (1969) and Shih and Bonner (1970b) have shown that, if there is an α-helical structure from residues 1 to 16 and from 20 to 23, then all of the cationic side chains in cluster (*a*) are in proper position to interact with DNA phosphates. The lysine and arginine side chains assume extended conformations, as determined by X-ray crystallography (Marsh and Donohue, 1967). Residues 8, 12, 16, 18, and 20 are all separated by equal distances, while the spacing between residues 3 and 8 is only slightly longer, and these distances are compatible with the spacing of phosphates on a given DNA strand. Residues 17, 19, and 23 are also separated by the same distance and can bind to the phosphates of the opposite DNA strand. Finally, by skipping two phosphate groups, residue 5 can bind to a third phosphate. On the basis of these studies, Shih and Bonner (1970b) proposed that the histone IV molecule can attach in the large groove of DNA and assume the necessary helical curvature, provided that one or two hydrogen bonds of the long α-helical segment are broken. The inner portion of the α-helix is composed principally of amino acids with short side chains, and it can therefore come into intimate contact with the DNA groove. It is worth noting that pairs of adjacent basic amino acids bind to phosphates of opposite DNA strands, and that this configuration occurs repeatedly in the histones. In addition, a spacing in which every fifth amino acid is a basic one is quite frequent.

Olins (1969) attempted to elucidate the site of histone I binding in a DNA–histone I complex reassociated in a salt gradient. He found

that the presence of histone I did not inhibit binding of actinomycin D in the small groove of DNA. He also reported that a histone I complex with nonglucosylated T2 DNA is a less effective substrate for glucosylation by α-glucosyl transferase than is such DNA alone. These results are consistent with the conclusion that histone I binds in the large groove of the DNA double helix.

C. Selective Dissociation

1. *Methods*

The general technique for selective dissociation of histones from chromatin involves brief exposure of the chromatin to a dissociating agent, followed by separation of the partially dehistonized chromatin from the dissociated proteins and the reagent by differential sedimentation or gel filtration.

2. *Structural Modifications*

Samples of chromatin which have been partially dehistonized by increasing concentrations of sodium chloride (NaCl) or sodium deoxycholate (DOC) are more heterogeneous in protein distribution than native chromatin or pure DNA. This is indicated by an increase in the band width of formaldehyde-fixed, partially dehistonized samples when centrifuged in a cesium chloride density gradient (Ilyin and Georgiev, 1969; Brutlag *et al.*, 1969; Farber, 1970). Such samples also have an increased heterogeneity in charge distribution when compared with native chromatin or pure DNA, as shown by an increased electrophoretic dispersion (band width) exhibited after free-zone electrophoresis (Ohlenbusch *et al.*, 1967; Smart and Bonner, 1970a; Levinson *et al.*, 1970). To provide additional evidence that a sample of partially dehistonized chromatin is composed of many discrete, slightly different types of molecule, Smart and Bonner (1970b) fractionated such samples by precipitation with 0.15 *M* NaCl. In this reagent, chromatin remains soluble or it aggregates, depending on the effective charge of the chromatin molecules. The authors found that partially dehistonized chromatin can be separated into fractions with different histone/DNA ratios, demonstrating that partial removal of histones does not uniformly alter all chromatin molecules. Consequently, measurements of any biophysical or biochemical property of a partially dehistonized chromatin sample probably represent average values for individual, discretely different nucleoprotein molecules in the sample.

The DNA in native chromatin differs in conformation from free DNA, as shown by an increased absorbance at 260 mμ and a reduced hyperchromicity upon melting (Doty *et al.*, 1960; Tuan and Bonner, 1969). A gradual increase in melting hyperchromicity has been observed in samples from which increasing amounts of total histone were removed by sodium chloride (Tuan and Bonner, 1969; Smart and Bonner, 1970), by guanidine hydrochloride (Levinson *et al.*, 1970), or by sodium deoxycholate (Smart and Bonner, 1970a). If this increase in melting hyperchromicity represents a loss of the conformation characteristic for DNA in native chromatin, then this conformation does not appear to depend on any one histone class.

Several investigators have studied the thermal denaturation of partially dehistonized chromatin (Ohba, 1966b; Ohlenbusch *et al.*, 1967; Levinson *et al.*, 1970; Smart and Bonner, 1970a,b). The T_m decreases as increasing amounts of histone are removed, while the melting profiles become considerably broader; however, the curves are not cleanly divided into DNA-like and native chromatin-like regions. Smart and Bonner (1970b) found that removal of histone by NaCl generates a nucleoprotein which has a lower T_m than that generated by removal of equivalent histone weight fractions by DOC. At the same time, if the fractional decrease in T_m is compared with the fraction of histone net positive charge removed from the DNA, the two methods of histone dissociation yield essentially identical curves. It appears that different histone fractions do not vary greatly in their ability to stabilize DNA against thermal denaturation, provided that a correction is made for differences in net positive charge among the fractions.

The sedimentation coefficients of sheared chromatin and of the DNA obtained from it have been compared (Ohba, 1966b; Chalkley and Jensen, 1968; Jensen and Chalkley, 1968; Smart and Bonner, 1970b). There seems to be general agreement that most methods of shearing purified chromatin ($s_{20,w} > 200$ S) produce molecules sedimenting between 25 and 30 S. The DNA purified from this 30 S chromatin sediments with an $s_{20,w}$ between 14 and 16 S, corresponding to a molecular weight of about 3×10^6. Since DNA purified from unsheared chromatin exhibits an $s_{20,w}$ of about 24 S, it appears that the shearing of purified chromatin involves a scission of the DNA contained in it. That the various histones do not differ greatly with regard to increasing the S value of chromatin is suggested by the studies of Ohba (1966b) and of Chalkley and Jensen (1968) on chromatin partially dehistonized by increasing concentrations of NaCl, and of Smart and Bonner (1970b) using both DOC and NaCl as the dissociating agents.

Native DNA exhibits a strong flow dichroism; this is expected for

the B-form of the Watson-Crick double helix, in which the planes of the bases are perpendicular to the helix axis. Because the absorbance of histone at 259 mμ is only 1.7% that of DNA (Ohba, 1966b), the contribution of the histones themselves can be neglected in measurements of flow dichroism. Ohba (1966b) and Smart and Bonner (1970b) found that the flow dichroism of native chromatin is 20 to 30% that of the DNA isolated from it. This decrease indicates both a decreased orientability of the DNA due to shortening of the whole molecule (presumably by supercoiling), and a decrease in the number of base pairs perpendicular to the axis of the oriented molecule. In order to eliminate the effect of the greater orientability of DNA molecules, Ohba (1966b) sheared the DNA to a size having a rotatory diffusion constant approximately equal to that of chromatin. When he then measured differences in flow dichroism, presumably reflecting only the altered orientation of DNA base pairs in chromatin (presumably due to supercoiling), he found that the number of base pairs perpendicular to the axis of the chromatin molecule is on the average 40% of that of the base pairs in fragmented DNA.

Available evidence indicates that the structure of chromatin is complex, probably involving several levels of organization, and that one of these levels includes supercoiling of chromatin fibers. One model which has been discussed is that of a DNA double helix (width approximately 30 Å with associated protein) which is supercoiled to yield a molecule of about 80 Å width and about 110 Å pitch (Pardon *et al.*, 1967; Moudrianakis, 1969; Griffith and Bonner, 1970b). This supercoil involves a calculated 40% shortening of the DNA double helix and tilts the planes of the bases to approximately a 35 degree angle with respect to the axis of the chromatin molecule.

Using flow dichroism techniques, Ohba (1966b) observed that the altered orientation of base pairs in chromatin partially dehistonized by increasing concentrations of NaCl largely depends on the less dissociable histones, i.e. histones II, III, and IV. Smart and Bonner (1970b) confirmed this discovery, but in addition they found that in chromatin partially dehistonized by increasing concentrations of DOC, the altered orientation of base pairs largely depends on histone I (which in this case is the less dissociable histone). Consequently, it appears that the altered flow dichroism of DNA in chromatin remains until the last portion of histone has been removed, and that it is inconsequential whether this last portion is histone I or some other fraction.

It has been suggested that histone I is a "cross-linker" of individual chromatin fibers (Littau *et al.*, 1965; Chalkley and Jensen, 1968); however, Smart and Bonner (1970b) found that the supposed cross-links

are irreversibly disrupted by extraction with 0.015 *M* DOC, which removes mostly histone II and no histone I. Therefore, if cross-linking is not an artifact, it does not seem to depend on any one histone class.

3. *Template Activity in Relation to Precipitation*

The ability of chromatin to act as a template for RNA synthesis *in vivo* or *in vitro* is greatly reduced as compared with pure DNA (Bonner and Huang, 1963; Littau *et al.*, 1964; Georgiev *et al.*, 1966; Paul and Gilmour, 1966). This template activity is an inverse function of the histone/DNA ratio of the chromatin (Bonner *et al.*, 1968b), and it can be restored to that of pure DNA by removal of histones by salt dissociation (Huang and Bonner, 1962; Marushige and Bonner, 1966). Sonnenberg and Zubey (1965) suggested that the reduced template activity of chromatin in the low ionic strength system described by Marushige and Bonner (1966) might have been due to aggregation of chromatin in the incubation medium. However, the template activity of chromatin is independent of its state of aggregation over a wide range of salt concentrations (0.05–0.3 *M*). At the salt concentration found in the nucleus (about 0.25 *M*), chromatin is probably aggregated. Recent results with *E. coli* RNA polymerase have suggested that a high ionic strength reaction mixture (0.2 *M*) may enable the enzyme to initiate transcription repeatedly (Millette and Trotter, 1970).

Smart and Bonner (1970c) studied the effect of selectively removing histones with increasing concentrations of NaCl or DOC on the ability of the partially dehistonized chromatins to support DNA-dependent RNA synthesis; the reactions were catalyzed by *E. coli* RNA polymerase in a high ionic strength medium (0.2 *M* KCl). They found that the template activity of the partially dehistonized samples was inversely proportional to the fraction of template covered by histone (Fig. 4). For example, the template activity increased from that of native chromatin (approximately 25% that of pure DNA in these experiments) to that of pure DNA, showing nearly a linear relationship to the amount of histone coverage of the template. Because the two methods used for selective removal of histones yielded entirely different patterns of histone dissociation, it is clear that the lowered template activity of native chromatin is not due solely to any one histone. Kinetic analysis of the data showed that the template concentration required to half-saturate the enzyme was constant (Smart, 1970). It therefore appears that *E. coli* RNA polymerase has access to aggregated chromatin templates.

Georgiev *et al.* (1966) found that the increase in template activity which they observed after extraction of chromatin with 0.6 *M* NaCl

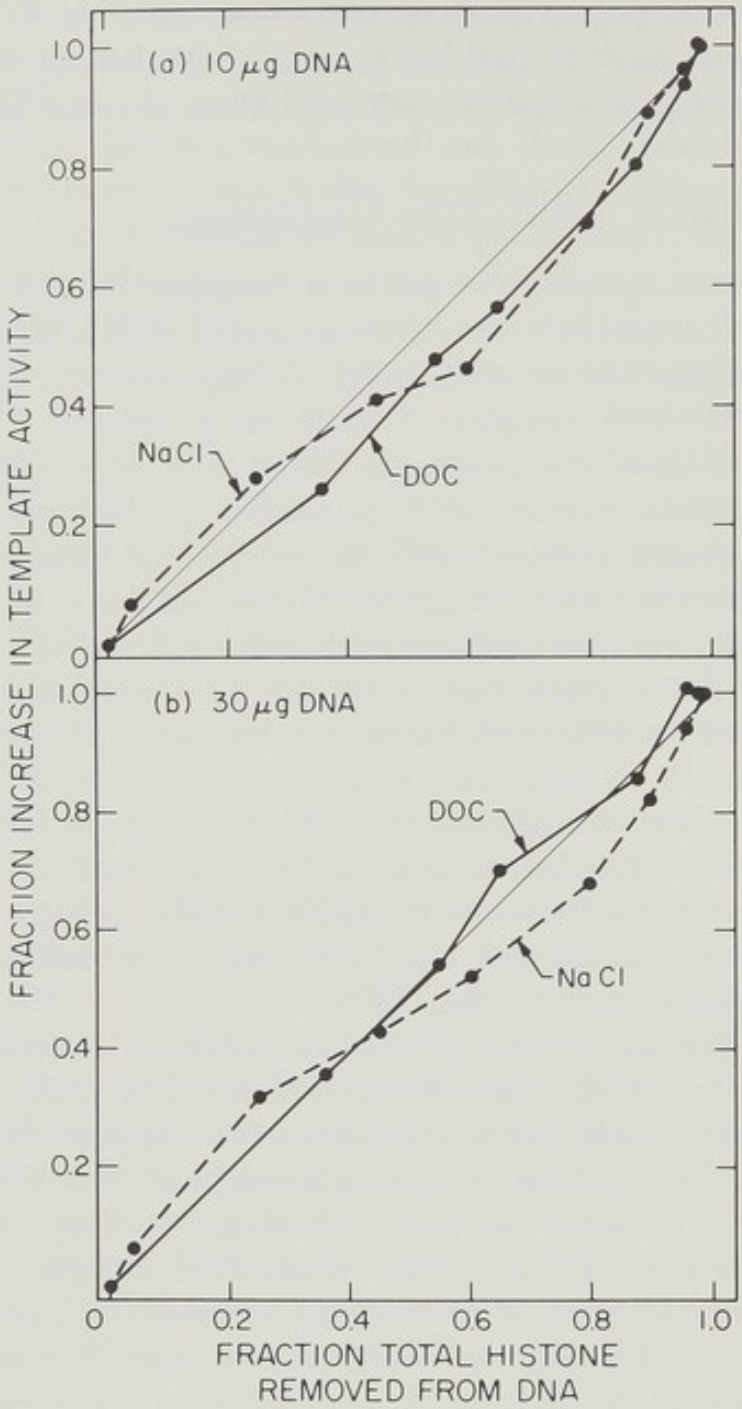

FIG. 4. (a) The fraction increase in template activity (10 μg DNA/0.25 ml assay solution) versus the fraction total histone removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl. The template activity of native chromatin is assigned the value of 0, while that of chromatin completely dehistonized by 3.0 *M* NaCl or 0.15 *M* DOC is assigned the value of 1.00. (b) Same as (a) except that template concentration was 30 μg/0.25 ml assay solution (Smart and Bonner, 1970).

could not be attributed to an increase in the solubility of the nucleoprotein because its solubility remained very low. Furthermore, Smart and Bonner (1970c) found that removal of a given weight fraction of

total histone by a given NaCl concentration produced a nucleoprotein which was more soluble in 0.15 *M* NaCl than the sample produced by removing an equal amount of total histone by DOC (Fig. 5A). Even after corrections were made for the net positive charge of each histone fraction, the greater solubility of chromatin samples partially dehistonized by NaCl was still apparent (Fig. 5B). It can be concluded that the template activity of a partially dehistonized sample for RNA synthesis in 0.2 *M* KCl is not closely related to its precipitability in 0.15 *M* NaCl. Nevertheless, both these salt concentrations are well within the limits for chromatin precipitation (0.05 to 0.30 *M* NaCl; Chalkley and Jensen, 1968).

There are several unanswered questions about the nature of the RNA transcribed from partially dehistonized templates. If NaCl or DOC remove histones from random sites on the chromatin, one would expect the incremental RNA transcribed *in vitro* to consist of sequences representing the entire genome. Although the results obtained by Georgiev *et al.* (1966) were consistent with this hypothesis, Bonner *et al.* (1968b) reported to the contrary. Much remains to be discovered in this area. There is, for example, the "read through" problem: Can RNA polymerase, once it has initiated transcription, read through a histone covered region? Another unresolved question concerns the mobility of the histones remaining after removal of a portion of the histone population: Can the remaining histone molecules shift, enlarging some gaps and diminishing others?

Smart and Bonner (1970c) called attention to several isolated facts suggesting that histone I is deposited on the DNA double helix in such a way that it is more exposed to the aqueous environment than the other histones. Especially in mammalian tissues, histone I is more susceptible to degradation by contaminating proteolytic enzymes than are the other histones (Fambrough, 1968; Panyim *et al.*, 1968). Furthermore, Brutlag *et al.* (1969) observed that histone I is the first histone fraction to be fixed to the DNA when chromatin is exposed to increasing concentrations of formaldehyde. Chalkley and Jensen (1968) observed that, when chromatin is precipitated by 0.15 *M* NaCl in the presence of DNA or RNA with molecular weights greater than 3×10^4, the extent of precipitation is greatly reduced; they showed that this is due to an increased negative charge on the chromatin molecules caused by migration of some histone I molecules to the free nucleic acid. Another piece of evidence is the well-established fact that histone I, which on the basis of its amino acid composition should have the strongest ionic interaction with DNA phosphate groups, is nevertheless the histone most readily dissociated by ionic agents. Smart and Bonner (1970c) concluded

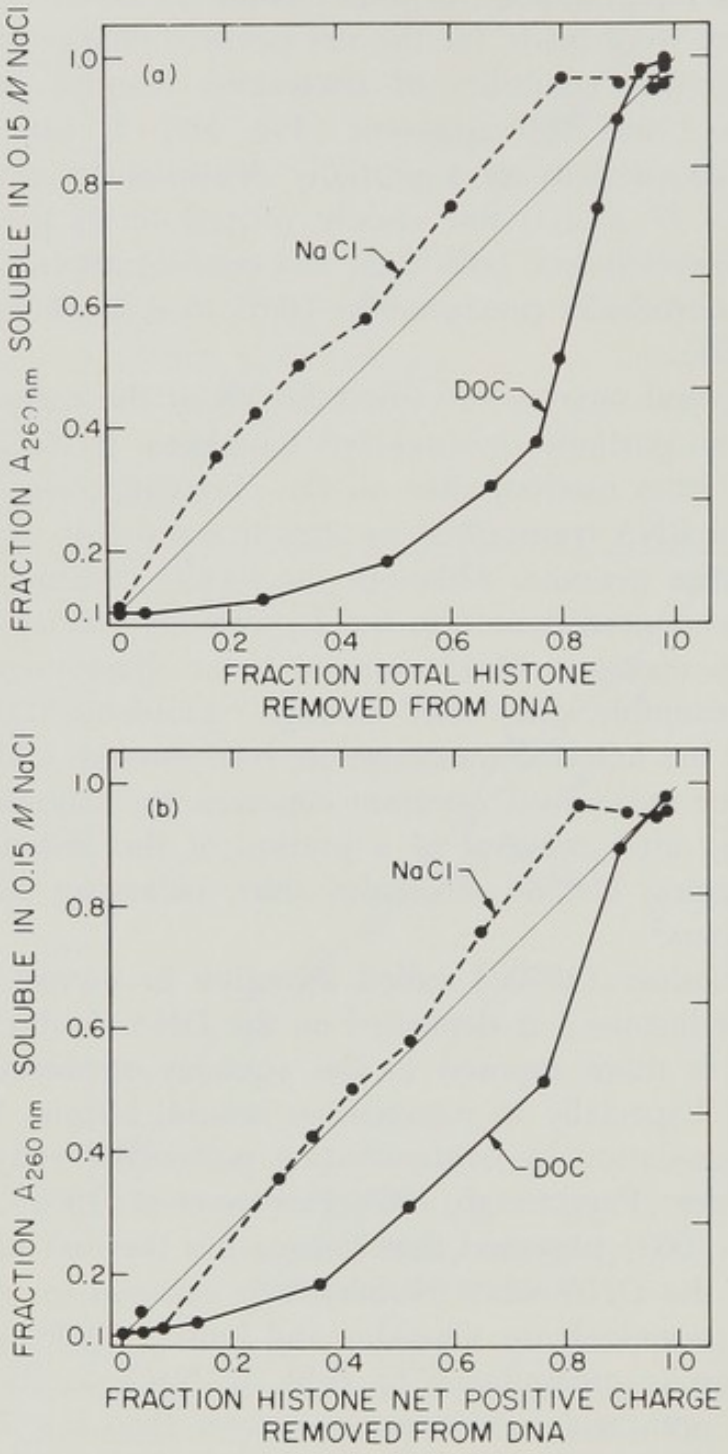

FIG. 5. (a) The fraction of $A_{260\,m\mu}$ material contained in chromatin which is soluble in 0.15 *M* NaCl–0.0025 *M* Tris, pH 8, versus the fraction of total histone removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl. (b) The fraction of $A_{260\,m\mu}$ material contained in chromatin which is soluble in 0.15 *M* NaCl–0.0025 *M* Tris, pH 8, versus the fraction of histone net positive charge removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl (Smart and Bonner, 1970).

that all these properties of histone I can be accounted for on the hypothesis that histone I is more exposed to and/or interacts more with the aqueous medium than do the other histones.

IV. Nonhistone Chromosomal Proteins

The nonhistone chromosomal proteins (NHC proteins) most probably include polymerases, nucleases, and other enzymes involved in the metabolism of chromatin, specific repressor or activator molecules, possibly structural proteins analogous to histones, and perhaps some nuclear membrane components. That this protein fraction acts at least partly in a regulatory fashion has been inferred from findings that the quantity of NHC protein is related to the type and physiological state of the tissue of origin (Dingman and Sporn, 1964; Bonner *et al.*, 1968b), from its relative abundance in euchromatin (Frenster, 1965) and metaphase chromosomes (Sadgopal and Bonner, 1970b), and from its high metabolic activity (Holoubek and Crocker, 1968). Chromosomal gels consisting of DNA and residual nonhistone proteins are disrupted by acid–urea extraction (which removes 60% of the protein) or by reagents which break disulfide bonds, implying that these proteins are important for the structure of chromatin (MacKay *et al.*, 1968; Hilgartner, 1968).

Nonhistone chromosomal proteins have not yet been studied extensively, primarily because of their tendency to aggregate with DNA, histones, and one another. There is also the problem of defining a "chromosomal" protein. For the purposes of this review, proteins prepared from nuclei or (preferably) chromatin, which can be shown to coprecipitate with histones and DNA from low concentrations of NaCl, will be considered chromosomal. Johns and Forrester (1969) showed that in 0.14 *N* NaCl, calf thymus deoxyribonucleoprotein absorbs acidic protein from cytoplasm or nuclear sap; approximately two thirds of these proteins seem to be removed by 0.35 *M* NaCl. By contrast, when chromatin prepared by the method of Bonner *et al.* (1968a) is extracted with 0.3 *N* NaCl, only 10% of the protein is removed, and this is principally nonhistone (Smart and Bonner, 1970). The fraction of NHC protein removed is enriched in the higher molecular weight polypeptides (Elgin, unpublished observation). Presumably all such NHC proteins are in dynamic equilibrium with the chromatin complex in the nucleus, so the question of the degree of association with DNA *in vivo* is somewhat academic as long as cytoplasmic contamination is ruled out.

A. Preparations

Benjamin and Gellhorn (1968) described a method for preparing nuclear acidic proteins from rat and mouse liver. Purified nuclei were extracted with 0.15 *M* NaCl + 0.01 *M* Na_2 EDTA and with 0.1 *M* HCl to remove ribonucleoprotein and histones respectively. The acidic nonhistone protein and DNA were then extracted with 4 *M* CsCl at pH 11.6 (lysine buffer) and separated by equilibrium density centrifugation. The recovered protein was dialyzed and concentrated in 0.01 *M* lysine, pH 11.6, 4 *M* deionized urea, 0.002 *M* Na_2 EDTA, and 0.005 *M* β-mercaptoethanol. The resulting solution of acidic proteins, when examined for chemical characteristics and heterogeneity by gel electrophoresis, appeared to include phosphoproteins. At pH 11 the protein sedimented as a single band with a sedimentation coefficient of 2.7 S. It is possible that the high pH necessary to extract this protein fraction may alter it.

Holoubek and Crocker (1968) prepared an acidic protein fraction from Ehrlich ascites cell nuclei and studied the synthesis of this fraction relative to RNA synthesis. Nuclei were extracted to remove acid-soluble proteins and RNA, after which an 0.5 *M* hot perchloric acid extract removed the DNA together with closely associated acidic proteins. Some protein remained associated with this DNA on CsCl density gradient centrifugation. Labeling experiments with radioisotopes suggested that the acidic protein fraction represents a mixture of proteins labeled independently. These proteins seem to be synthesized in the usual way, but have a higher specific activity when DNA-like RNA is being made.

Wang (1967) also studied the isolation and fractionation of chromosomal acidic proteins. Rat liver nuclei were isolated, washed with Mg-Tris buffer to remove soluble proteins, and the chromatin was then solubilized with 1 *M* NaCl. The salt solution extract was dialyzed against 0.14 *M* NaCl, precipitating the DNA together with such proteins as were bound to it. Those proteins remaining in the supernatant were considered to be chromatin acidic proteins; they were further fractionated by ammonium sulfate precipitation and by acid precipitation at pH 5.7 and pH 4.8. Such protein fractions proved to be very heterogeneous, as judged by starch gel electrophoresis; furthermore, the ammonium sulfate fraction included a protein with an isoelectric point greater than pH 8.5. Acidic proteins prepared in this way sedimented with DNA in dilute salt solutions and coprecipitated with histone. Each fraction was further purified by DEAE cellulose chromatography at pH 8.2. In all cases there was a protein peak eluting at approximately 0.2–0.3 *N* NaCl, a peak which eluted at 0.5–0.6 *N* NaCl, and a significant run-off

peak (suggesting histone contamination) (Wang and Johns, 1968). If chromatin purified according to the method of Bonner *et al.* (1968a) is precipitated from 0.15 *N* NaCl and centrifuged at about 12,000 *g*, the proteins of the supernatant include not only the whole population of nonhistone chromosomal protein but also small amounts of histone; this is because centrifugation under these conditions does not pellet all of the chromatin. Wang's preparation may well have included these as well as other nuclear proteins.

A fourth means of preparing nonhistone chromosomal protein was developed by Marushige *et al.* (1968). Chromatin isolated by the method of Bonner *et al.* (1968a) was acid extracted to remove histones, and the residue was then solubilized in 1% sodium dodecyl sulfate (SDS) and 0.05 *M* Tris at pH 8. Following dialysis against 0.1% SDS and 0.01 *M* Tris, the DNA was removed from the solution by centrifugation at 106,000 *g* for 18 hours. The proteins in the supernatant were recovered by ammonium sulfate precipitation or used directly for chemical analyses and gel electrophoresis. In the presence of 0.1% SDS, proteins prepared in this way have an S value of 2.7 and an average molecular weight of 14,300; however, in the absence of detergent the protein aggregates to higher molecular weight forms. It is likely that the standard protein preparation still has detergent associated with it. Because nonhistone proteins isolated by this procedure can be examined directly by SDS gel electrophoresis (Shapiro *et al.*, 1967), it is a very useful method for determining the heterogeneity of nonhistone chromosomal proteins and for comparing the nonhistone protein populations of various tissues. Although acid extraction of chromatin may seem to be a drastic treatment, SDS electrophoresis of native chromatin dissolved in SDS indicates the same population of nonhistone proteins found after preparation by this method (Elgin and Bonner, 1970). Shirey and Huang (1969) also used SDS to prepare a total chromosomal protein fraction; however, solubility problems were encountered as the detergent was removed.

Recently a method of fractionating chromosomal proteins by selective coprecipitation with polyethylene sulfonate (PES) has been developed (Krauze *et al.*, 1969). Chromatin was dissociated in 5 *M* urea and 2 *N* NaCl, after which DNA was removed by ultracentrifugation. When a 20-fold dilution was made in the presence of PES at pH 7, a PES–histone complex precipitated. By contrast, the nonhistone proteins precipitated with PES at pH 4. The latter fraction appeared similar to Wang's as judged by disc gel electrophoresis (MacGillivray *et al.*, 1969), but the fractionation was incomplete and degradation-aggregation problems were apparent (Cameron *et al.*, 1969).

Chromosomal proteins can be fractionated by dissociating chromatin

in 5 *M* urea–2 *M* NaCl, removing nucleic acid by centrifugation, and separating histones from NHC proteins by ion exchange chromatography, hydroxylapatite columns, etc. However, the methods described to date are unsatisfactory because the yield of NHC proteins is low, cross contamination with histones occurs, the proteins aggregate, etc. (Cameron *et al.*, 1969; Elgin, unpublished observation). A phosphoprotein fraction can also be prepared from "chromatin acidic proteins" by fractionation with calcium-phosphate gel (Langan and Smith, 1966; Kleinsmith and Allfrey, 1969). Unfortunately, these proteins rapidly aggregate and are difficult to handle (Gershey and Kleinsmith, 1969).

B. Heterogeneity

The degree of heterogeneity of nonhistone chromosomal protein fractions has been a matter of some controversy. The electrophoretic patterns, centrifugation data, and enzymatic activities of chromatin acidic proteins, as prepared by the Wang procedure, all suggest that this fraction is highly heterogeneous (Wang, 1967). On the other hand, residual proteins from chromatin prepared by the Dounce procedure (Dounce and Hilgartner, 1964; MacKay *et al.*, 1968), when tested by disc or paper electrophoresis, seem to consist predominantly of two polypeptide chains. Acidic nuclear proteins prepared by the Benjamin and Gellhorn (1968) procedure, analyzed by polyacrylamide gel electrophoresis, contain 12 to 15 polypeptide chains at a minimum. Furthermore, the band pattern is similar for preparations from rat liver, mouse liver, and euchromatin from rat liver nuclei (Benjamin and Gellhorn, 1968). The *in vivo* labeling patterns of DNA-associated proteins prepared by Holoubek and Crocker (1968) from Ehrlich ascites cells indicated a mixture of proteins labeling independently of one another. Since disc gel polyacrylamide electrophoresis is a limited technique, in which conditions are subjectively chosen to show only the dominant proteins, it can be concluded that the nonhistone chromosomal protein fraction probably contains from 12 to 20 different polypeptide chains (Elgin and Bonner, 1970).

Elgin and Bonner (1970) did a comparative study of chromosomal nonhistone proteins prepared by a modification of the technique of Marushige *et al.* (1968). Using SDS disc gel electrophoresis, they found that the protein patterns of rat kidney and rat liver are nearly the same (Fig. 6). Of the twelve major bands, ten were identical for the two tissues. However, they differed in that liver possesses bands κ and λ, whereas in this region kidney possesses only band ω. Presumably SDS

disc gel electrophoresis fractionates only according to molecular weights (Shapiro *et al.*, 1967).

The gel electrophoresis pattern of nonhistone proteins from pea bud chromatin differs considerably from that of rat liver. Although most of the lower molecular weight bands run like those from liver, the homology breaks down in the middle and higher molecular weight regions. By contrast, chromosomal nonhistone proteins from chicken liver preparations are quite similar to those of rat liver preparations. Chicken erythrocytes, from which the chromatin has little template activity for RNA synthesis, yields a high molecular weight protein not observed in any of the other tissues. The lower molecular weight bands β and

FIG. 6. Nonhistone chromosomal proteins of rat liver (bottom) and rat kidney by SDS disc gel electrophoresis. Gels run from left to right (Elgin and Bonner, 1970).

γ have been found in all tissues examined so far. Comparative studies of acidic nuclear proteins prepared by a modification of Wang's method have yielded analogous results in that the proteins of homologous tissues from different vertebrates seem to be similar (these preparations have included histone I). Chick erythrocyte preparations showed two dominant protein bands (Loeb and Creuzet, 1969). These results are consistent with the view that the nonhistone chromosomal proteins include various enzymes of chromatin metabolism common to all tissues, as well as proteins that are tissue specific. Further chemical and functional studies of these proteins are needed.

C. Interaction with Histones and DNA

One of the reasons for interest in the NHC proteins is the possibility that they may be part of a mechanism which introduces sequence spec-

ificity into histone-DNA interactions; such specificity may also depend on associated chromosomal RNA (Bekhor *et al.*, 1969; Huang and Huang, 1969). A number of investigations have suggested that nonhistone chromosomal proteins may be involved in gene activation. However, in order to demonstrate that NHC proteins produce biologically significant derepression in an *in vitro* RNA synthesizing system, it must be shown that (1) the observed effects cannot be explained by a simple coprecipitation of histone; (2) the nonhistone protein is associated with the DNA; (3) the effect occurs at the normal salt concentrations of the nucleus, i.e. approximately 0.2 *M* (Langendorf *et al.*, 1961); (4) it is the chromatin which is affected and not exogenous RNA polymerase; (5) proteases have not been added; and (6) the derepression is specific. Although a critical experiment has not been reported, it has been observed that nonhistone chromosomal proteins coprecipitate with histones (Marushige *et al.*, 1968; Wang and Johns, 1968). Such coprecipitation is most pronounced at low salt concentrations, such as those frequently used for RNA polymerase assays. A nonhistone phosphoprotein fraction isolated from rat liver nuclei by Langan (1967) also forms insoluble complexes with histones at low salt concentrations.

Early studies indicated that, when histones are selectively removed from chromatin, the resulting complex containing most of the NHC proteins has a template activity equal to pure DNA (Marushige and Bonner, 1966). Furthermore, when DNA and SDS-prepared nonhistone chromosomal proteins are mixed at high salt concentrations and then allowed to associate by dialysis to low salt concentrations, NHC proteins bind to the DNA but the reconstituted material is as effective a template for RNA synthesis as deproteinized DNA (Marushige *et al.*, 1968). On the other hand, Gilmour and Paul (1969) discovered that a reconstituted template (salt-urea dialysis) consisting of DNA, histone, and acidic protein has the template activity of chromatin, while a reconstituted template consisting of DNA and histone alone has no template activity. This ability of acidic protein to inhibit the restriction of DNA template activity by histones is not a unique property of the NHC proteins; in the experiments of Gilmour and Paul (1969) bovine serum albumin performed as well or better. However, the NHC proteins seem to be necessary if the reconstitution is to be sequence specific in terms of particular RNA sequences transcribed (as judged by hybridization competition). It has been shown that these NHC protein fractions contain RNA (Cameron *et al.*, 1969), and this RNA may be identical with that shown to play a role in specific reconstitution of chromatin (Bekhor *et al.*, 1969; Huang and Huang, 1969).

Wang also found that his acidic chromosomal protein fraction could

significantly reduce the amount of inhibition by exogenous histone of an *in vitro* RNA synthesis system (DNA template). The degree of derepression depended on the histone used for inhibition, and the order in which components were added had no effect (Wang, 1968a). Wang's preparation also reversed histone repression of DNA synthesis, apparently by binding the histone competitively (Wang, 1969).

Teng and Hamilton (1969) demonstrated that the addition of nonhistone protein to a mixture synthesizing RNA from a calf endometrium chromatin template reverses inhibition by added histone. In this system the order in which protein components were added had a definite effect, but some mitigation of histone inhibition was achieved even when the histone was added prior to the NHC protein. Acidic nuclear proteins from other tissues were also effective. Complexes containing nuclear phosphoprotein and histone cause some inhibition of DNA-dependent RNA synthesis *in vitro*, yet are not as effective as histones alone (Langan, 1967; Langan and Smith, 1966). Spelsberg and Hnilica (1969) reported that acidic nuclear proteins, including the phosphoprotein preparation of Langan (1967), prevent histone inhibition of DNA-dependent RNA synthesis *in vitro* only when they interact together prior to the addition of DNA template. They obtained similar results with a nuclear acidic protein fraction prepared by the method of Wang (1967). These observations are consistent with the idea that NHC proteins do not in themselves repress template activity but that they do interact with histones and/or DNA to mitigate the repression by histones. Although there is some evidence that NHC proteins may also act as repressors, definitive controls are lacking (Paul and Gilmour, 1968). Caution is necessary because acid extraction of chromatin may not only remove histones but also damage the remaining structure, leading to a change in template activity and melting profile (Bannai and Terayama, 1969). Neither a *specific* derepression or specific repression by NHC proteins has been demonstrated so far.

D. Enzymatic Activities

A number of enzyme activities have been found in nonhistone chromosomal or acidic nuclear proteins. Among the best established is a neutral protease isolated and characterized by Furlan and Jericijo (1967a,b). Calf thymus nuclei were lysed and the pellet treated with 1 *N* NaCl, extracting the protease activity with the chromatin. The purified protease has a molecular weight of about 24,000, a pH optimum of 7.8, and a maximum activity in 0.1 to 1 *M* NaCl. It is inhibited by *p*-chloromer-

curibenzoate and by diisopropylfluorophosphate, suggesting that there is a serine residue at the active site. This protease shows some specificity for deoxyribonucleohistone (see Section V,B). That the enzyme is normally a constituent of chromatin is indicated by the fact that it coprecipitates with DNA and histones at NaCl concentrations between 0.1 and 0.3 *N*. Furthermore, as shown by these reconstitution and acid extraction experiments, the binding between the protease and DNA seems to be quite similar to that between DNA and histone I (Furlan and Jericijo, 1967a,b; Furlan *et al.*, 1968). The characteristics of this enzyme suggest that it may be responsible for part or all of the histone degradation observed by Panyim *et al.* (1968) and others.

Other enzymes involved in histone modification might be expected in chromatin. There is protein phosphokinase activity in a phosphoprotein preparation from acidic nuclear proteins (analogous to Wang, 1967; see Langan, 1967; Kleinsmith and Allfrey, 1969). Comb *et al.* (1966) found a methylase producing ϵ-*N*-methyllysine in histones, which is probably a chromosomal protein (see also Sekeris *et al.*, 1967).

A number of enzymes concerned with nucleic acid metabolism are associated with chromatin. The presence of aggregate RNA polymerase in chromatin is well established (EC 2.7.7.6; Weiss, 1960; Huang *et al.*, 1960). This may be related to a soluble RNA polymerase not associated with chromatin, as prepared by several investigators (Seifart and Sekeris, 1969; Liao *et al.*, 1968). It has been suggested that only half of the total RNA polymerase is firmly associated with nuclear chromatin and/or participates in active RNA synthesis (Liao *et al.*, 1968). DNA polymerase (EC 2.7.7.7) has also been purified from a Wang preparation of acidic chromosomal proteins derived from rat liver and calf thymus. The acidic proteins of chromatin show a higher specific activity per milligram protein than do other fractions such as the nuclear sap (Patel *et al.*, 1967). Chromatin DNA polymerase shows optimal activity in the presence of all four deoxyriboside 5′-triphosphates and has an absolute requirement for template DNA and divalent metal ions, particularly Mg^{++}. It is active with templates from a variety of sources, but shows the greatest activity with homologous rat liver nuclear DNA (Howk and Wang, 1969a). Although at low enzyme/DNA ratios its preferred template is native DNA, at higher enzyme/DNA ratios its preferred template is heat-denatured DNA. This difference does not seem to be the consequence of endonuclease activity (Howk and Wang, 1970b). Some preparations may contain two DNA polymerases, one with a preference for each type of template (Howk and Wang, 1969b). In any case, the reaction product consists of small, heterogeneous DNA fragments (Howk and Wang, 1970a). Preparations may also include

some terminal transferase activity, an enzyme which has been reported in a similar fraction from calf thymus (Wang, 1968b).

A neutral deoxyribonuclease (possibly EC 3.1.4.5) has been observed in the isolated nuclei of mammalian cells and remains associated with chromatin prepared by the method of Paul and Gilmour (1966), i.e., precipitated from 0.2 *M* sodium phosphate buffer. However, preparations of soluble nucleohistone prepared by the method of Zubay and Doty (1959) were not active. This enzyme is of the deoxyribonuclease-1 type, the products being 3′-hydroxyl terminated fragments of DNA (Swingle *et al.*, 1967). O'Connor (1969) has also isolated a deoxyribonuclease from rat liver acidic chromosomal proteins (Wang method), which functions predominantly as an endonuclease and may be identical with that reported above. A comparison of the template preferences of DNA polymerase and deoxyribonuclease isolated from the acid protein fraction and from the nuclear sap shows that enzymes with different template preferences are present in different amounts in the two fractions. Consequently, O'Connor (1969) has suggested that some of these enzymes may be chromosomal in the sense of being largely bound to chromatin *in vivo*, while others are not.

There have also been numerous reports of NAD nucleosidase activity (EC 3.2.2.5) associated with chromatin (e.g., Bock *et al.*, 1968). In addition, Wang (1967) showed that his acidic protein fraction contains glutamate dehydrogenase (EC 1.4.1.2), glutamic oxaloacetic transaminase (EC 2.6.1.1), lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), and ATPase (EC 3.6.1.3). The significance of these enzymes is unknown.

V. Metabolism of Chromosomal Proteins

A. Synthesis

Metabolic studies on the histones have yielded several general conclusions. In most cells the histone proteins seem to be metabolically stable; furthermore, they are synthesized in the usual way, with most of the synthesis occurring simultaneously with DNA replication. However, the exact site of synthesis is still controversial. In various rat tissues histones and DNA have equal half-lives (Byvoet, 1966). In the onion root meristem, Bloch *et al.* (1967) showed that much of the histone is conserved from one cell division to the next. Similarly, Hancock (1969) detected no significant irreversible dissociation of histones from chromatin during several mitotic divisions in mouse mastocyte PB 15 cells. This low rate of histone turnover is good evidence for the metabolic

integrity of the deoxyribonucleoprotein complex, even though small amounts of turnover would not be detected in most studies. Unlike histones, the NHC proteins seem to undergo turnover at a rate similar to that of cytoplasmic proteins.

That synthesis of chromosomal proteins occurs by the usual ribosomal mechanism has been well established. Several groups have shown that histone synthesis is inhibited by puromycin (Deisseroth, 1969; Allfrey *et al.*, 1964a). Furthermore, Borun *et al.* (1967) isolated small polyribosomes from HeLa cell cytoplasm which were associated with nascent polypeptides of high lysine and low tryptophan content. These polysomes were present only in the S phase of the cell cycle, and disappeared on treatment with cytosine arabinoside, an inhibitor of histone and DNA synthesis. Synthesis of a 7–9 S RNA precedes histone and DNA synthesis by 1 hour; this presumed histone messenger has been isolated and shown to have a half-life of about 1 hour (as compared with 3 hours for most mRNA). Histones have also been synthesized *in vitro* on cytoplasmic microsomes from HeLa cells (Gallwitz and Mueller, 1969a,b); the products were identified by their co-electrophoresis with native histones on urea polyacrylamide gels. Kedes and Gross (1969) isolated three RNA species from the cytoplasm of cleaving sea urchin embryos, suggesting that there were mRNAs for histones. In these embryos, half the proteins synthesized in the cytoplasm accumulated in the nucleus, mostly on the chromosomes. The sea urchin 9–10 S RNA species were found in light polysomes and decreased in quantity on treatment of the embryo with hydroxyurea (which has an effect analogous to that of cytosine arabinoside in HeLa cells). Histone-specific mRNAs should have a relatively high adenine content (Baer, 1964), but the nucleotide composition of the presumptive RNAs has not yet been determined. Nemer and Lindsay (1969) reported isolating slowly sedimenting polysomes from sea urchins (200 S); these polysomes accumulated as synthesis of chromosomal proteins increased, and they incorporated arginine actively in contrast to low tryptophan incorporation (tryptophan does not occur in histones).

These various lines of evidence strongly support the view that histones are synthesized by the usual ribosomal mechanism in the cytoplasm. However, there is evidence that histones may be synthesized in the nucleus as well. Incorporation of amino acids into histones by a puromycin-sensitive mechanism has been demonstrated in isolated nuclei (Allfrey *et al.*, 1964a; Reid and Cole, 1964; Trevithick, 1969). Both of these experiments involved nuclei from thymocytes, which contain intranuclear ribosomes and the machinery for synthesizing proteins (Allfrey, 1963).

Most histone synthesis occurs concurrently with the synthesis of DNA, i.e., during the S phase of the cell cycle. This was first shown in *Euplotes eurystomus* (Prescott, 1966), and since then has been confirmed in *Tetrahymena pyriformis* (Hardin *et al.*, 1967), mouse ascites tumor (Yarbro, 1967), onion root meristem (Bloch *et al.*, 1967), Ehrlich ascites tumor (Lederer and Sandritter, 1967), HeLa cells (Robbins and Borun, 1967; Sadgopal, 1968), primary spermatocytes of *Urechis caupo* (Das and Alfert, 1968), and regenerating rat liver (Takai *et al.*, 1968). However, Butler and Cohn (1963) reported that histone synthesis precedes DNA synthesis in partially hepatectomized rats, and similar results have been reported for bovine sex chromosomes (Herzog and Steffensen, 1968) and for mitotic insect cells (Bogdanov *et al.*, 1968); these may be special cases. In some cells histone synthesis continues after DNA synthesis has been inhibited (Flamm and Birnstiel, 1964; Spalding *et al.*, 1966; Gurley and Hardin, 1968).

Although a burst of histone synthesis coincides with DNA synthesis, histones turn over at other times in some tissues. In calf endometrium, Chalkley and Maurer (1965) found that histones III and IV were synthesized at a rate approximating that of whole cytoplasmic protein, while histones I and II were synthesized only upon DNA replication. By contrast, Dick and Johns (1969a) found that the various rat thymus histones turned over at similar rates, and they attributed an apparently slower turnover of histone I to the presence of contaminating nonhistone protein. Synthesis of arginine-rich histones without concomitant DNA synthesis has been reported in chicken erythrocytes (Sadgopal and Kabat, 1969). In HeLa cells, Spalding *et al.* (1966) reported independent metabolic patterns for the individual histone fractions, with most synthesis occurring at the time of DNA synthesis; similar results were obtained by Sadgopal and Bonner (1969). Evidently some histone synthesis depends on concurrent DNA synthesis, while some continues in its absence. Sadgopal and Bonner (1969) suggested that in slowly dividing, highly differentiated cells histone and DNA synthesis are coupled, while in rapidly proliferating undifferentiated cells (such as L cells or HeLa cells) at least part of the histone synthesis is independent of DNA synthesis. However, even in the latter case initiation of DNA synthesis leads to a marked increase in the rate of histone synthesis.

B. Degradation

Little work has been done either on the mechanisms for intracellular degradation of histone and NHC proteins, or on the way these proteins are removed from chromatin *in vivo*. This is unfortunate, because specific

removal of histones is implied in many theories of gene regulation. Degradation of histones is also a problem during purification of chromatin. Phillips and Johns (1959) reported that chromatin contains a proteolytic enzyme capable of degrading histone molecules; this protease was active at neutral and alkaline pH values and was inhibited by 1 m*M* diisopropylfluorophosphate (DFP). A subsequent detailed study of the process was carried out by Panyim *et al.* (1968), who followed the degradation of isolated nucleohistone by the decrease in its precipitability in 0.15 *M* NaCl; they observed a 20% degradation within 1 day at 0°C. Although DNA isolated from this chromatin appeared to be unchanged, the histone yield decreased dramatically. Histone I appeared to be most susceptible to protease degradation. Stellwagen *et al.* (1968) also found that histone I is degraded when isolated nuclei are incubated at 37°C. Isolated histones are fairly stable (Vidali and Neelin, 1968).

A careful investigation of isolated rat liver nuclei by Dounce and associates suggested the presence of several proteases with different pH optima (Dounce and Umana, 1962; Umana and Dounce, 1964; Dounce *et al.*, 1966; Dounce and Ickowicz, 1969). Furlan and Jericijo (1967a) discovered that calf thymus deoxyribonucleoprotein contains two histone splitting proteases which have maximal activities at pH 4.4 and 7.8, respectively. Analysis of the coprecipitation of these proteases with chromatin from 0.15 *M* NaCl suggested that the acid protease may have been a cytoplasmic contaminant, but the neutral protease is associated with chromatin *in vivo* (Furlan and Jericijo, 1967b). Histone I is most susceptible to this neutral protease. By comparing the amount of various histones which remain following incubation of deoxyribonucleoprotein for 20 hours with the crude enzyme preparation either at 37° or at 0°, the authors found that 52% of histone f1, 32% of histone f2, and 19% of histone f3 were degraded (Furlan and Jericijo, 1967a). Under optimal conditions of salt concentration and pH, they also found that deoxyribonucleoprotein is six to seven times more susceptible to the partially purified protease than are globular proteins (Furlan *et al.*, 1968). It is possible that this neutral protease plays a role in genetic control mechanisms, particularly those involving the removal of histones on a large scale. It would be interesting to know if the enzyme preferentially degrades phosphorylated histones (see Section V,E). However, this protease may be largely inactive *in vivo*, inasmuch as numerous studies indicate a very low rate of histone turnover in the normal cell or nucleus (see Section V,A). In fact, Furlan and Jericijo (1967b) observed an absolute increase in the neutral protease activity following purification by gel filtration and suggested that a normal inhibitor had been removed.

C. Changes in Histone Distribution during Growth and Development

As discussed in Section II,C, many of the tissues in adult vertebrates contain the same histones. Although specific histones do not generally appear during embryogenesis, or in response to hormones, etc., significant quantitative changes in the proportions of the various histones are sometimes observed. For example, chromatin from different embryonic and adult chick tissues has the same total histone/DNA ratio within experimental error (Dingman and Sporn, 1964); furthermore, these embryonic and adult histones have the same properties by starch gel electrophoresis and amino acid analysis (Kischer and Hnilica, 1967). Although careful study of histones from early chick embryos between gastrulation and 7 days of age revealed some quantitative differences in histone proportions, no essential qualitative or large quantitative differences were found. All the adult histones were present in the earliest embryos examined (Kischer *et al.*, 1966). When developing embryonic tissues were followed between days 2 and 16, changes in proportions of histones were detected, leading to those typical of the adult organs (Lindsay, 1964; Agrell and Christensson, 1965). Similar results have been found for bird erythrocytes (Champagne and Mazen, 1969). Few studies have been done on histone changes during aging. However, a comparison of young calf and aged cow thymus has shown that there is considerably less arginine-rich histone in the cow thymus (Pyhtila and Sherman, 1968).

Cytochemical investigations of events during and immediately following fertilization have suggested that the cleavage nuclei and subsequent cells up to the blastoderm stage may contain "juvenile" histones. The chromosomal protein in these instances stains as a weakly basic protein (Das *et al.*, 1964; Vaughn, 1968). However, such observations are difficult to interpret, and could be due either to histone phosphorylation and similar modifications, or to a relatively low level of histone in many pregastrula embryos (Asao and Ishida, 1969). Analysis of histones in sea urchin blastula and subsequent stages by gel electrophoresis has shown that juvenile histones, if they exist, occur only in minor quantities (Marushige and Ozaki, 1967; Vorobyev *et al.*, 1969). A few investigations on developing plants have also indicated only quantitative histone differences between the dormant and germinating states (Gofshtein, 1968; Teraoka, 1968). In maturing pea cotyledons the same spectrum of histones occurs throughout, except that there is an increasing amount of histone I (Fambrough *et al.*, 1968).

The histone/DNA ratio is evidently correlated with chromosomal tem-

plate activity, such that chromatin of higher template activity has a lower histone/DNA ratio (Bonner *et al.*, 1968b). However, there are no gross differences between the histones of interphase, prophase, or metaphase cells of higher organisms (Stocken and Ord, 1966; Maio and Schildkraut, 1967; Comings, 1967; Sadgopal and Bonner, 1970b). Although a unique histone has been reported in meiotic tissues in lilies, characterized by gel electrophoresis (Sheridan and Stern, 1967), there is a possibility that this is histone III linked by disulfide bridges to another protein. Some recent careful studies have also suggested histone differences related to the mitotic activity of tissues. For example, Chalkley and Panyim (1969) reported that calf tissues essentially lacking in cell division, i.e., heart, brain, lung, kidney, and liver, contain a minor histone not present in rapidly dividing tissues such as thymus or intestinal mucosa. Likewise, the latter tissues have a minor histone not present in the others. These results may be due to microheterogeneity (Sections D and E), but amino acid analysis of the characteristic minor histone from slowly replicating tissues indicates that this is a unique, lysine-rich histone (Panyim and Chalkley, 1969b). Quantitative but not qualitative differences in the histone complement have been observed in rapidly proliferating tissues such as HeLa and Morris hepatoma relative to normal tissues (Sadgopal and Bonner, 1970a; Vescia *et al.*, 1967; Laurence *et al.*, 1963).

Bird erythrocytes contain a characteristic minor histone (Section I); this component is not related solely to the genetic inactivity of erythrocyte chromatin, since it is also present in the histones of genetically active bird reticulocytes and erythroblasts, i.e., cells which contain ribosomes and make mRNA (Dick and Johns, 1969b). Other comparisons of genetically active and inactive chromatin derived from a single tissue have failed to reveal any qualitative difference in histone types (Comings, 1967; Gorovsky and Woodard, 1967) or in lysine/arginine ratio (Berlowitz, 1965).

In some species the population of histones associated with DNA changes radically during the course of spermatogenesis. In these examples the somatic histones are replaced by protamine or by proteins apparently more basic than the normal somatic histones. The process evidently involves a replacement of histones by protamines (Marushige *et al.*, 1969), rather than a conversion of histones to more basic molecules as has been occasionally suggested (Gledhill *et al.*, 1966). Bloch (1969) has provided a detailed recent review on this subject.

Several investigators have analyzed changes in histones following hormonal induction of a tissue. Frequently the induced target tissue shows increased RNA synthesis *in vivo* and/or increased chromatin template

activity *in vitro* [e.g., induction of immature rabbit uterus with estrogen (Cohen *et al.*, 1964); induction of rat liver with hydrocortisone (Dahmus and Bonner, 1965); induction of tadpole liver chromatin by thyroxine (Kim and Cohen, 1966)]. However, the expected changes in histone/DNA ratio, if they occur, are too small to be detected accurately by present analytical methods. At the same time it is evident that changes in template activity caused by hormonal induction must be related to protein complement, since DNA isolated from induced and control chromatins is equally efficient as a template (Dahmus and Bonner, 1965; Kim and Cohen, 1966). Stellwagen and Cole (1968) reported changes in the amounts of particular histones after hormonal induction of mammary glands. Although the three major histone classes were not different in kind or amount from animals in different stages of pregnancy and lactation, the pattern of subclasses within the lysine-rich histones did show significant variation (Stellwagen and Cole, 1968, 1969a). Differences in the incorporation of labeled amino acids into these histone I subfractions were also observed in induced and noninduced mammary tissue cultures (Hohmann and Cole, 1969); however, the rate of incorporation into the lysine-rich fraction as a whole was not altered (Marzluff *et al.*, 1969). Hormonal response in this and a few other systems (but not in all) appears to require cell division (Stockdale and Topper, 1966); this relationship may mean that gene derepression is achieved not by histone removal but by simply avoiding histone binding during replication.

D. Methylation and Acetylation of Histones

Chemical modification of histones following polypeptide chain synthesis has been demonstrated in a number of systems. It has been suggested that such modifications of histone molecules may alter their association with DNA, changing the structure of the chromatin and leading to a different phenotypic state of the cell. The most important chemical modifications of this kind are methylation, acetylation, and phosphorylation.

That plant and animal histones undergo methylation *in vivo* is now well established, and this process apparently can be duplicated *in vitro;* such methylation occurs after polypeptide synthesis (Allfrey *et al.*, 1964b; Kim and Paik, 1965; Sekeris *et al.*, 1967). Protein methylation activity is widely distributed (Paik and Kim, 1969a). In many systems, arginine-rich histones are methylated preferentially, but exceptions have been reported (Comb *et al.*, 1966; Sekeris *et al.*, 1967; Orenstein and Marsh, 1968; Patterson and Davies, 1969; Kaye and Sheratzky, 1969). Both lysine and arginine residues undergo methylation, probably

catalyzed by different enzymes (Paik and Kim, 1967b, 1968). Furthermore, the turnover of methyl groups can be very rapid, especially in the lysine-rich histones (Paik and Kim, 1967b). Tidwell *et al.* (1968) showed that methylation of histones is a relatively late event in regenerating rat liver, occurring after derepression and after DNA and histone synthesis. As yet the biological significance of such methylation is unknown, but it is suggestive that polymers of dimethyllysine show increased resistance to proteolysis (Seeley and Benoiton, 1969).

Histones can be acetylated both at the N-terminal residue and at residues within the molecule (Phillips, 1961, 1963; Gershey *et al.*, 1968). Acetylating enzymes have been prepared from a wide variety of systems, and some have been shown to acetylate arginine-rich histones preferentially (Allfrey *et al.*, 1964b; Vidali *et al.*, 1968). This acetylation also occurs after polypeptide synthesis (Gallwitz, 1968; Gallwitz and Sekeris, 1969; Nohara *et al.*, 1966, 1968). In calf thymus histone IV, only lysine 16 is acetylated (to approximately 50%) while in pea histone IV lysine 16 and at least one of the lysines at 5, 8, and 12 are acetylated (DeLange *et al.*, 1969b). In addition, deacetylase has been described from calf thymus cells, but its specificity for histones remains to be determined (Inoue and Fujimoto, 1969). Acetyl groups on interior lysine residues of histones III and IV turn over fairly rapidly (Vidali *et al.*, 1969).

Although it is well established that histones are acetylated to a small extent *in vivo*, the degree to which this acetylation alters their association with DNA is uncertain. Allfrey *et al.* (1964b) found that synthetically acetylated histones (which can reassociate with DNA *in vitro*) are not as effective as native histones in repressing RNA synthesis by isolated calf thymus nuclei. On the other hand, Clark and Byvoet (1969) reported that histones fully acetylated at their N-terminal residues are just as effective as nonacetylated histones in repressing RNA synthesis *in vitro*. Both groups of investigators evidently agreed that acetylation of histones does not significantly prevent their binding with DNA.

Growth and differentiation have been examined in a number of tissues to establish a time course for histone acetylation in relation to other cellular changes. There is a significant increase in acetyl content of the arginine-rich histones, particularly IV, in regenerating rat liver within the first few hours following hepatectomy. This change does not seem to be due to differential synthesis or degradation of histones, and it precedes or coincides with increased RNA polymerase activity and RNA synthesis. These observations do not establish a causal link between the various events (Pogo *et al.*, 1968, 1969). In human and equine lymphocytes the rate of RNA synthesis increases within minutes after ex-

posure to phytohemagglutinin (PHA), while at the same time the rate of histone acetylation but not of histone synthesis increases greatly. By contrast, PHA inhibits both RNA synthesis and histone acetylation in equine granulocytes (Pogo *et al.*, 1966, 1967). As the authors pointed out, these temporal relationships in no way establish a causal relationship among the events, and histone acetylation may be a nonspecific effect of PHA in the lymphocyte system (Ono *et al.*, 1969; MacGillivray and Monjardino, 1968). In rapidly dividing tissues of calf, histone IV is acetylated at lysine residue 16 about half the time, but much less so in slowly dividing tissues (Panyim and Chalkley, 1969a). The sum of the evidence at hand suggests that histone acetylation may be an early nonspecific event in chromatin derepression. An excellent detailed review of this subject has been provided by Allfrey (1969).

E. Phosphorylation

Phosphorylation of histones has also been proposed as a mechanism for regulating their repressor activity. Histone phosphorylation is an energy-dependent process that occurs in cell nuclei independently of polypeptide synthesis (Kleinsmith *et al.*, 1966). Most of the phosphate is incorporated into histones and NHC proteins as phosphoserine, although a small amount of phosphothreonine has also been detected (Stevely and Stocken, 1966; Kleinsmith *et al.*, 1966; Benjamin and Goodman, 1969). In many cases, the phosphate groups turn over rapidly, and dephosphorylation may also be energy dependent (Kleinsmith *et al.*, 1966). In addition, phosphorylation of some nuclear acidic proteins has been observed (Benjamin and Gellhorn, 1968).

Several enzyme preparations have been isolated which are capable of phosphorylating histones. For example, a protein kinase which catalyzes the transfer of phosphate from ATP to serine residues of thymus histones, or to salmon and herring protamines, has been purified from rat liver. All histones can serve as substrates for this enzyme, but f1 and f2b histones are most active. The enzyme has been separated from phosphoprotein kinase and does not phosphorylate nonhistone proteins (Langan and Smith, 1967). Judging from differences in substrate preference among different enzyme fractions, there is probably more than one such histone kinase (Langan, 1968b). Meisler and Langan (1967, 1969) also reported the isolation of a histone phosphatase from rat liver, for which phosphorylated histone f1 and protamine were the preferred substrates. The reaction products were the intact protein and orthophosphate. The purified enzyme had a maximum reaction rate at

pH 7–8 with ionic strength 0.1–0.2, approximately the conditions within cell nuclei. Histone phosphatase activity has been found in all rat tissues examined, as well as in chick embryo, pea seedling, *Neurospora*, *Euglena*, and other eukaryotes; however it has not been found in bacteria or in blue-green algae.

Phosphorylation of chromosomal proteins may be related to alterations in gene expression. Stevely and Stocken (1966, 1968b) observed that phosphorylated histone f1, when added to an *in vitro* RNA-synthesizing system, was not as effective an inhibitor as nonphosphorylated f1. Furthermore, phosphorylation of nuclear proteins is an early event in the response of lymphocytes to PHA (Kleinsmith *et al.*, 1966). The extent of histone phosphorylation in different tissues was studied by Gutierrez and Hnilica (1967), who examined the ratio of phosphate incorporation to lysine incorporation in liver, regenerating liver, Novikoff hepatoma, and spleen. Histone fractions were phosphorylated to different extents, with the lysine-rich histones highest. Their data indicate that histone phosphorylation decreases with increasing tissue mitotic rates; however, differences in precursor pool size were not taken into account and could change the interpretation. Autoradiographic studies of dipteran salivary gland chromosomes have revealed phosphorus incorporation into NHC protein both in puffed regions and in regions without puffs (Benjamin and Goodman, 1969).

Langan (1968a, 1969b) studied the effects of hormones on histone phosphorylation; liver histone kinase increased in activity by 8-fold and 20-fold, respectively, after intraperitoneal injections of adenosine 3′,5′-cyclic monophosphate or of N^6, $O^{2'}$-dibutyryl cyclic AMP. Administration of glucagon *in vivo* caused a 10- to 20-fold increase in phosphorylation of histone I of rat liver. The major peptide of histone f1 which was phosphorylated in these experiments was the same as that involved during *in vitro* phosphorylation by the purified histone kinase, indicating that a specific serine is the site of phosphorylation (Langan, 1969a,b).

Recent studies on mouse mammary epithelial cells in organ culture have also shown that hormonal induction of these cells to divide and differentiate is accompanied by an increase in the phosphorylation of histones and nuclear acidic proteins concomitant with an increase in RNA synthesis. Although previously synthesized polypeptide chains appear to be the phosphorylation substrates, the site of the reaction and the dependency of the events are unknown (Turkington and Riddle, 1969). Variable phosphate incorporation into histone f1 subclasses has been reported for rat thymus nuclei labeled *in vitro* (Buckingham and Stocken, 1969).

In studies of histone phosphorylation, special care is required to avoid

contamination by lipids, nonhistone phosphoproteins, RNA, DNA, and their precursors. Shepherd *et al.* (1970) recently showed that nonhistone protein, RNA, and DNA can accompany some histones through extensive purification procedures, and this contamination is likely to be misleading in labeling experiments. Quantitative studies of phosphate incorporation into histone should be based on estimates of *O*-phosphoserine and *O*-phosphothreonine.

Histone phosphorylation may be an important process in cell division. For example, histone f1 seems to be phosphorylated to a greater extent in mitotically active tissues (Stevely and Stocken, 1968a). In addition, the amount of nuclear nonhistone phosphoprotein has been directly correlated with the mitotic index for several tissues (Langan, 1967). The time course of histone f1 phosphorylation in regenerating rat liver is similar to that of DNA synthesis (Ord and Stocken, 1967; Stevely and Stocken, 1968b), and histone f1 kinase activity changes in a corresponding manner (Pawse *et al.*, 1969). Incorporation of phosphate into histone f1 is depressed by irradiation in a pattern similar to the dose response curve for DNA synthesis. However, this effect may be due to an alteration in the ATP metabolism of the nucleus (Ord and Stocken, 1966b). Unilaterally nephrectomized rats show an increase in f1 phosphorylation concomitant with an increase in DNA synthesis, and the effect of gamma irradiation on this response is parallel for both reactions (Stevely and Stocken, 1968a,b). A similar increase in histone f1 phosphate during DNA synthesis occurs in regenerating pancreas (Stocken and Ord, 1969). These observations are consistent with the possibility that histone phosphorylation is a necessary part of the transport mechanism by which newly synthesized histones move from the cytoplasm into the nucleus.

Dixon and his collaborators have studied the phosphorylation of basic proteins during spermatogenesis in trout (Dixon *et al.*, 1969a). They established that protamine is synthesized by the usual ribosomal mechanism in the cytoplasm, where it is subsequently phosphorylated, probably as *O*-phosphoserine. Several phosphopeptides were recovered from labeled preparations, and apparently all the newly incorporated serine residues were phosphorylated. However, in mature spermatozoa the serine residues of protamine are not phosphorylated, indicating a displacement of the phosphate. The initial serine phosphorylation may be necessary for transport of protamine from cytoplasm to nucleus (Trevithick *et al.*, 1967; Ingles and Dixon, 1967).

All histone fractions in this system are also phosphorylated, apparently *in situ* on the DNA (Marushige *et al.*, 1969). Later, histones are displaced from the DNA, with histone I the last to leave. Histones II

and IV contain only one phosphate-labeled peptide, i.e. the N-terminal tripeptide *N*-acetyl-*O*-phosphorylserylglycylarginine. Histones I and III have characteristic and different phosphopeptide patterns. The phosphorylation, like those previously discussed, is independent of protein synthesis (Dixon *et al.*, 1969b; Marushige and Dixon, 1969).

An enzyme has been isolated from trout testis which phosphorylates histones and protamines; it is Mg^{++} dependent, thiol dependent, and is stimulated by cyclic 3′,5′-AMP. In 0.3 *M* NaCl it shows a 13.5 times preference for protamine over whole histone (Jergil and Dixon, 1970). Interestingly enough, dissociation of phosphorylated histone from testis chromatin by increasing concentrations of salt or of detergent essentially parallels the dissociation of nonphosphorylated histones; this suggests that phosphohistones may not be significantly different in their affinity for DNA (Marushige *et al.*, 1969), leading to the speculation that histone phosphorylation may serve as a signal for specific proteases in the cell nucleus.

VI. Conclusions

Sufficient data are now in hand to reevaluate the role of the histones. The number of histone species is small. The near perfect conservation of the histone IV amino acid sequence during evolution is unprecedented. Histone IV must perform an essential function, interacting with other highly conserved molecular structures such as the DNA sugar-phosphate backbone. Other histones have been conserved to a lesser extent. Although definitive experiments are lacking, there is nothing at this time to suggest that histones recognize specific base sequences. However, the N-terminal half of histone IV appears well constructed to interact with DNA in a manner analogous to the protamines.

These facts suggest that molecules other than histones must lend specificity to the mechanisms of gene repression. Selective removal experiments using NaCl (and other salts) or DOC to remove histones in a different order show that the different histone fractions generally have comparable effects; all histones contribute to the stability of the chromatin structure and all contribute to the repression of the genome. On the other hand, histone I appears to be exceptional in many ways, and the histone fractions do change in relative amounts during growth and development; the subtle utilization of these changes to control gene structure and expression is not yet fully understood. Further studies on the regulatory role of histones should concentrate on their interaction with other components of the chromatin which could dictate specificity.

Few conclusions can be reached concerning the nonhistone chromo-

somal proteins, and even their definition is difficult. The fraction is heterogeneous, comprising 12–20 major polypeptide chains and doubtless many others in small amounts. Some of these major proteins appear to be tissue specific. In addition, several enzyme activities have been purified from chromatin. Still, the role of these proteins in the control of chromosome replication and gene expression remains obscure.

REFERENCES

Agrell, I. P. S., and Christensson, E. G. (1965). *Nature (London)* **207**: 638.

Allfrey, V. G. (1963). *Exp. Cell Res. Suppl.* **9**: 183.

Allfrey, V. G. (1969). *In* "Biochemistry of Cell Division" (R. Baserga, ed.), p. 179. Thomas, Springfield, Illinois.

Allfrey, V. G., Littau, V. C., and Mirsky, A. E. (1964a). *J. Cell Biol.* **21**: 213.

Allfrey, V. G., Faulkner, R., and Mirsky, A. E. (1964b). *Proc. Nat. Acad. Sci. U.S.* **51**: 786.

Ando, T., and Suzuki, K. (1966). *Biochim. Biophys. Acta* **121**: 427.

Ando, T., and Suzuki, K. (1967). *Biochim. Biophys. Acta* **140**: 375.

Ando, T., and Watanabe, S. (1969). *Int. J. Protein Res.* **1**: 221.

Ando, T., Iwai, K., Ishii, S. I., Azegami, M., and Nakahara, C. (1962). *Biochim. Biophys. Acta* **56**: 628.

Asao, T., and Ishida, J. (1969). *In* "Histones and Gene Function" (K. Iwai, ed.), p. 137. The Research Group on Histones and Gene Function in Japan.

Baer, D. (1964). *J. Theor. Biol.* **6**: 282.

Bannai, S., and Terayama, H. (1969). *J. Biochem. (Tokyo)* **66**: 289.

Bekhor, I., Kung, G. M., and Bonner, J. (1969). *J. Mol. Biol.* **39**: 351.

Bellair, J. T., and Mauritzen, C. M. (1964). *Aust. J. Biol. Sci.* **17**: 990.

Bellair, J. T., and Mauritzen, C. M. (1967). *Biochim. Biophys. Acta* **133**: 263.

Benjamin, W. B., and Gellhorn, A. (1968). *Proc. Nat. Acad. Sci. U.S.* **59**: 262.

Benjamin, W. B., and Goodman, R. M. (1969). *Science* **166**: 629.

Berlowitz, L. (1965). *Proc. Nat. Acad. Sci. U.S.* **54**: 476.

Billen, D., and Hnilica, L. S. (1963). *J. Cell Biol.* **19**: 7a.

Black, J. A., and Dixon, G. H. (1967). *Nature (London)* **216**: 152.

Blazek, V., and Bukaresti, L. (1964). *Experientia* **20**: 369.

Bloch, D. P. (1969). *Genetics* **61** (Suppl.): 1.

Bloch, D. P., Macquigg, R. A., Brack, S. D., and Wu, J.-R. (1967). *J. Cell Biol.* **33**: 451.

Bock, K., Gang, V., Beer, H. P., Kronav, R., and Grunicke, E. (1968). *Eur. J. Biochem.* **4**: 357.

Bogdanov, Y. F., Liapunova, N. A., Sherudilo, A. I., and Antropova, E. N. (1968). *Exp. Cell Res.* **52**: 59.

Bonner, J., and Huang, R. C. C. (1963). *J. Mol. Biol.* **6**: 169.

Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C. C., Huberman, J. A., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B. M., and Widholm, J. (1968a). *Methods Enzymol.* **12**: 3.

Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C. C., Marushige, K., and Tuan, D. Y. H. (1968b). *Science* **159**: 47.

Borun, T. W., Scharff, M. D., and Robbins, E. (1967). *Proc. Nat. Acad. Sci. U.S.* **58**: 1977.

Boulanger, P. A., Jaume, F., Moschetto, Y., and Biserte, G. (1969). *FEBS Lett.* **4**: 291.
Brutlag, D., Schlehuber, C., and Bonner, J. (1969). *Biochemistry* **8**: 3214.
Buckingham, R. H., and Stocken, L. A. (1969). *Biochem. J.* **114**: 55P.
Bustin, M., and Cole, R. D. (1968). *Arch. Biochem. Biophys.* **127**: 457.
Bustin, M., and Cole, R. D. (1969a). *J. Biol. Chem.* **244**: 5286.
Bustin, M., and Cole, R. D. (1969b). *J. Biol. Chem.* **244**: 5291.
Bustin, M., Rall, S. C., Stellwagen, R. H., and Cole, R. D. (1969). *Science* **163**: 391.
Butler, J. A. V., and Cohn, P. (1963). *Biochem. J.* **87**: 330.
Butler, J. A. V., Johns, E. W., and Phillips, D. M. P. (1968). *Progr. Biophys. Mol. Biol.* **18**: 209.
Byvoet, P. (1966). *J. Mol. Biol.* **17**: 311.
Cameron, A., Gilmour, R. S., Hill, E., Ingles, C. J., Krauze, R. J., MacGillivray, A. J., Paul, J., and Threlfall, G. (1969). Personal communication.
Chalkley, G. R. (1969). Personal communication.
Chalkley, G. R., and Jensen, R. (1968). *Biochemistry* **7**: 4380.
Chalkley, G. R., and Maurer, H. R. (1965). *Proc. Nat. Acad. Sci. U.S.* **54**: 498.
Chalkley, G. R., and Panyim, S. (1969). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**: 842.
Champagne, M., and Mazen, A. (1969). *Abstr. Commun. 6th FEBS Meet., Madrid* Abstr. No. 720.
Clark, P. R., and Byvoet, P. (1969). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**: 843.
Cohen, S. N., Spicer, S. S., and Yielding, K. L. (1964). *Biochim. Biophys. Acta* **87**: 511.
Cohn, N. S., and Quatrano, R. S. (1965). *Experientia* **21**: 237.
Comb, D. G., Sarkar, N., and Pinzino, C. J. (1966). *J. Biol. Chem.* **241**: 1857.
Comings, D. E. (1967). *J. Cell Biol.* **35**: 699.
Comings, D. E., and Kakefuda, T. (1968). *J. Mol. Biol.* **33**: 225.
Cozcolluela, C., and Subirana, J. A. (1968). *Biochim. Biophys. Acta* **154**: 242.
Cruft, H. J. (1961). *Biochim. Biophys. Acta* **54**: 611.
Cruft, H. J., and Leaver, J. L. (1961). *Nature (London)* **192**: 556.
Dahmus, M. E., and Bonner, J. (1965). *Proc. Natl. Acad. Sci. U.S.* **54**: 1370.
Das, C. C., Kaufmann, B. P., and Gay, H. (1964). *J. Cell Biol.* **23**: 423.
Das, N. K., and Alfert, M. (1968). *Exp. Cell Res.* **49**: 51.
Dayhoff, M. O., and Eck, R. V. (1968). "Atlas of Protein Chemistry 1967–68," pp. 33–45. Nat. Biomed. Res. Found., Silver Spring, Maryland.
Deisseroth, A. (1969). *Biochim. Biophys. Acta* **186**: 392.
DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1968a). *Proc. Nat. Acad. Sci. U.S.* **61**: 1145.
DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1968b). *J. Biol. Chem.* **243**: 5906.
DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1969a). *J. Biol. Chem.* **244**: 319.
DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1969b). *J. Biol. Chem.* **244**: 5669.
Desai, L., Ogawa, Y., Mauritzen, C. M., Taylor, C. W., and Starbuck, W. C. (1969). *Biochim. Biophys. Acta* **181**: 146.
Dick, C., and Johns, E. W. (1969a). *Biochim. Biophys. Acta* **174**: 380.

Dick, C., and Johns, E. W. (1969b). *Biochim. Biophys. Acta* **175**: 414.
Dick, C., and Johns, E. W. (1969c). *Comp. Biochem. Physiol.* **31**: 529.
Dingman, C. W., and Sporn, M. B. (1964). *J. Biol. Chem.* **239**: 3483.
Dixon, G. H., Ingles, C. J., Jergil, B., Ling, V., and Marushige, K. (1969a). *Can. Cancer Res.* p. 76.
Dixon, G. H., Marushige, K., Ling, V., Sung, M., and Wigle, D. T. (1969b). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**: 599.
Doty, P., Marmur, J., Eigner, J., and Schildkraut, C. (1960). *Proc. Nat. Acad. Sci. U.S.* **46**: 461.
Dounce, A. L., and Hilgartner, C. A. (1964). *Exp. Cell Res.* **36**: 228.
Dounce, A. L., and Ickowicz, R. (1969). *Arch. Biochem. Biophys.* **131**: 210.
Dounce, A. L., and Umana, R. (1962). *Biochemistry* **1**: 811.
Dounce, A. L., Seaman, F., and MacKay, M. (1966). *Arch. Biochem. Biophys.* **117**: 550.
Dove, W. F., Jr., and Davidson, N. (1962). *J. Mol. Biol.* **5**: 467.
Dwivedi, R. S., Dutta, S. K., and Bloch, D. P. (1969). *J. Cell Biol.* **43**: 51.
Edwards, L. J., and Hnilica, L. S. (1968). *Experientia* **24**: 228.
Elgin, S. C. R., and Bonner, J. (1970). *Biochemistry* in press.
Fambrough, D. M. (1968). Ph.D. Thesis, California Inst. of Technol., Pasadena, California.
Fambrough, D. M., and Bonner, J. (1966). *Biochemistry* **5**: 2563.
Fambrough, D. M., and Bonner, J. (1968a). *Biochim. Biophys. Acta* **154**: 601.
Fambrough, D. M., and Bonner, J. (1968b). *J. Biol. Chem.* **243**: 4434.
Fambrough, D. M., and Bonner, J. (1969). *Biochim. Biophys. Acta* **175**: 113.
Fambrough, D. M., Fujimura, F., and Bonner, J. (1968). *Biochemistry* **7**: 575.
Farber, M. (1970). Personal communication.
Flamm, W. G., and Birnstiel, M. L. (1964). *Exp. Cell Res.* **33**: 616.
Frenster, J. H. (1965). *Nature (London)* **206**: 680.
Froehner, S. C. (1969). Personal communication.
Furlan, M., and Jericijo, M. (1967a). *Biochim. Biophys. Acta* **147**: 135.
Furlan, M., and Jericijo, M. (1967b). *Biochim. Biophys. Acta* **147**: 145.
Furlan, M., Jericijo, M., and Suhar, A. (1968). *Biochim. Biophys. Acta* **167**: 154.
Gallwitz, D. (1968). *Biochem. Biophys. Res. Commun.* **32**: 117.
Gallwitz, D., and Mueller, G. C. (1969a). *Science* **163**: 1351.
Gallwitz, D., and Mueller, G. C. (1969b). *J. Biol. Chem.* **244**: 5947.
Gallwitz, D., and Sekeris, C. E. (1969). *Hoppe-Seyler's Z. Physiol. Chem.* **350**: 150.
Ganesan, A. T., and Lederberg, J. (1965). *Biochem. Biophys. Res. Commun.* **18**: 824.
Georgiev, G. P., Anaieva, L. N., and Kozlov, J. V. (1966). *J. Mol. Biol.* **22**: 365.
Gershey, E. L., and Kleinsmith, L. J. (1969). *Biochim. Biophys. Acta* **194**: 331.
Gershey, E. L., Vidali, G., and Allfrey, V. G. (1968). *J. Biol. Chem.* **243**: 5018.
Gershey, E. L., Haslett, G. W., Vidali, G., and Allfrey, V. G. (1969). *J. Biol. Chem.* **244**: 4871.
Gilmour, R. S., and Paul, J. (1969). *J. Mol. Biol.* **40**: 137.
Gledhill, B. L., Gledhill, M. P., Rigler, R., Jr., and Ringertz, N. R. (1966). *Exp. Cell Res.* **41**: 652.
Gofshtein, L. V. (1968). *Biokhimiya* **33**: 823.
Gorovsky, M., and Woodard, J. (1967). *J. Cell Biol.* **33**: 723.
Griffith, J. (1969). Personal communication.

Griffith, J., and Bonner, J. (1970a). In preparation.
Griffith, J., and Bonner, J. (1970b). In preparation.
Gurley, L. R., and Hardin, J. M. (1968). *Arch. Biochem. Biophys.* **128**: 285.
Gutierrez, R. M., and Hnilica, L. S. (1967). *Science* **157**: 1324.
Hallick, L., Boyce, R. P., and Echols, H. (1969). *Nature (London)* **223**: 1239.
Hancock, R. (1969). *J. Mol. Biol.* **40**: 457.
Hardin, J. A., Einem, G. E., and Lindsay, D. T. (1967). *J. Cell Biol.* **32**: 709.
Hearst, J., and Botchan, M. (1970). *Annu. Rev. Biochem.* **39**: 151.
Hempel, K., Lange, H. W., and Birkofer, L. (1968). *Naturwissenschaften* **55**: 37.
Herzog, R., and Steffensen, D. M. (1968). *Cytogenetics* **7**: 471.
Hilgartner, C. A. (1968). *Exp. Cell Res.* **49**: 520.
Hilton, J., and Stocken, L. A. (1966). *Biochem. J.* **100**: 21c.
Hnilica, L. (1966). *Biochim. Biophys. Acta* **117**: 163.
Hnilica, L., and Bess, L. G. (1965). *Anal. Biochem.* **12**: 421.
Hnilica, L., and Billen, D. (1964). *Biochim. Biophys. Acta* **91**: 271.
Hnilica, L., Johns, E. W., and Butler, J. A. V. (1962). *Biochem. J.* **82**: 123.
Hnilica, L., Taylor, C. W., and Busch, H. (1963). *Exp. Cell Res. Suppl.* **9**: 367.
Hohmann, P., and Cole, R. D. (1969). *Nature (London)* **223**: 1064.
Holoubek, V., and Crocker, T. T. (1968). *Biochim. Biophys. Acta* **157**: 352.
Howk, R., and Wang, T. Y. (1969a). *Arch. Biochem. Biophys.* **133**: 238.
Howk, R., and Wang, T. Y. (1969b). *Abstr. 158th Amer. Chem. Soc. Meet.* Abstr. No. 21. Biol. (ACS Journals, Wash. D.C.)
Howk, R., and Wang, T. Y. (1970a). *Arch. Biochem. Biophys.* **136**: 422.
Howk, R., and Wang, T. Y. (1970b). *Eur. J. Biochem.* **13**: 455.
Huang, R. C. C., and Bonner, J. (1962). *Proc. Nat. Acad. Sci. U.S.* **48**: 1216.
Huang, R. C. C., and Huang, P. C. (1969). *J. Mol. Biol.* **39**: 365.
Huang, R. C. C., Maheshwari, N., and Bonner, J. (1960). *Biochem. Biophys. Res. Commun.* **3**: 689.
Huang, R. C. C., Bonner, J., and Murray, K. (1964). *J. Mol. Biol.* **8**: 54.
Ilyin, Y. V., and Georgiev, G. P. (1969). *J. Mol. Biol.* **41**: 299.
Ingles, C. J., and Dixon, G. H. (1967). *Proc. Nat. Acad. Sci. U.S.* **58**: 1011.
Inoue, A., and Fujimoto, D. (1969). *Biochem. Biophys. Res. Commun.* **36**: 146.
Inoue, S., and Ando, T. (1968). *Biochem. Biophys. Res. Commun.* **32**: 501.
Iwai, K., Ishikawa, K., and Hayashi, H. (1969). *In* "Histones and Gene Function" (K. Iwai, ed.), p. 11. The Research Group on Histones and Gene Function in Japan.
Iwai, K., Ishikawa, K., Hayashi, H. (1970). *Nature* **226**: 1056.
Jellum, E. (1966). *Biochim. Biophys. Acta* **115**: 95.
Jensen, R. H., and Chalkley, G. R. (1968). *Biochemistry* **7**: 4388.
Jergil, B., and Dixon, G. H. (1970). *J. Biol. Chem.* **245**: 425.
Johns, E. W. (1964). *Biochem. J.* **92**: 55.
Johns, E. W. (1967). *Biochem. J.* **105**: 611.
Johns, E. W. (1968a). *J. Chromatogr.* **33**: 563.
Johns, E. W. (1968b). *Eur. J. Biochem.* **4**: 437.
Johns, E. W., and Butler, J. A. V. (1962). *Biochem. J.* **82**: 15.
Johns, E. W., and Butler, J. A. V. (1964). *Nature (London)* **204**: 853.
Johns, E. W., and Forrester, S. (1969). *Eur. J. Biochem.* **8**: 547.
Kawashima, S., Inoue, Sadako, and Ando, T. (1969). *Biochim. Biophys. Acta* **186**: 145.
Kaye, A. M., and Sheratzky, D. (1969). *Biochim. Biophys. Acta* **190**: 527.
Kedes, L. H., and Gross, P. R. (1969). *Nature (London)* **223**: 1335.

Kim, K. H., and Cohen, P. P. (1966). *Proc. Nat. Acad. Sci. U.S.* **55**: 1251.
Kim, S., and Paik, W. K. (1965). *J. Biol. Chem.* **240**: 4629.
Kinkade, J. M. (1969). *J. Biol. Chem.* **244**: 3375.
Kischer, C. W., and Hnilica, L. S. (1967). *Exp. Cell Res.* **48**: 424.
Kischer, C. W., Gurley, L. R., and Shepherd, G. R. (1966). *Nature (London)* **212**: 304.
Kleinsmith, L. J., and Allfrey, V. G. (1969). *Biochim. Biophys. Acta* **175**: 123.
Kleinsmith, L. J., Allfrey, V. G., and Mirsky, A. E. (1966). *Proc. Nat. Acad. Sci. U.S.* **55**: 1182.
Knippers, R., and Sinsheimer, R. L. (1968). *J. Mol. Biol.* **34**: 17.
Krauze, R. J., MacGillivray, A. J., and Paul, J. (1969). *Biochem. J.* **114**: 40P.
Langan, T. A. (1967). *In* "Regulation of Nucleic Acid and Protein Biosynthesis," Proc. Int. Symp., Lunteren, Neth. (V. V. Konigsberger and L. Bosch, eds.), p. 233. Elsevier, Amsterdam.
Langan, T. A. (1968a). *Science* **162**: 579.
Langan, T. A. (1968b). *In* "Regulatory Mechanisms for Protein Synthesis in Mammalian Cells" (A. San Pietro, M. R. Lamborg, and F. T. Kennedy, eds.), p. 101. Academic Press, New York.
Langan, T. A. (1969a). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**: 600.
Langan, T. A. (1969b). *J. Biol. Chem.* **244**: 5763.
Langan, T. A., and Smith, L. K. (1966). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **25**: 778.
Langan, T. A., and Smith, L. K. (1967). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **26**: 603.
Langendorf, H., Siebert, G., Lorenz, I., Hannover, R., and Beyer, R. (1961). *Biochem. Z.* **335**: 273.
Laurence, D. J. R., Simson, P., and Butler, J. A. V. (1963). *Biochem. J.* **87**: 200.
Leaver, J. L., and Cruft, H. J. (1966). *Biochem. J.* **101**: 665.
Lederer, B., and Sandritter, W. (1967). *Eur. J. Cancer* **3**: 21.
Lees, C. W., and von Hippel, P. H. (1968). *Biochemistry* **7**: 2480.
Leighton, T. J., Dill, B. C., and Stock, J. J. (1970). *Nature* in press.
Leng, M., and Felsenfeld, G. (1966). *Proc. Nat. Acad. Sci.* U.S. **56**: 1325.
Levinson, R. L., Smart, J. E., and Bonner, J. (1970). *Biochim. Biophys. Acta.* Submitted for publication.
Liao, S., Sagher, D., and Fang, S.-M. (1968). *Nature (London)* **220**: 1336.
Lindsay, D. T. (1964). *Science* **144**: 420.
Littau, V. C., Allfrey, V. G., Frenster, J. H., and Mirsky, A. E. (1964). *Proc. Nat. Acad. Sci. U.S.* **52**: 93.
Littau, V. C., Burdick, C. J., Allfrey, V. G., and Mirsky, A. E. (1965). *Proc. Nat. Acad. Sci. U.S.* **54**: 1204.
Loeb, J. E., and Creuzet, C. (1969). *FEBS Lett.* **5**: 37.
Luck, J. M., Rasmussen, P. S., Satake, K., and Tsvetikov, A. N. (1958). *J. Biol. Chem.* **233**: 1407.
MacGillivray, A. J. (1968). *Biochem. J.* **110**: 181.
MacGillivray, A. J., and Monjardino, J. P. P. V. (1968). *Biochem. J.* **108**: 22P.
MacGillivray, A. J., Ingles, C. J., and Krauze, R. J. (1969). *Abstr. Commun. 6th FEBS Meet., Madrid* Abstr. No. 230.
MacKay, C. A., Hilgartner, C. A., and Dounce, A. L. (1968). *Exp. Cell Res.* **49**: 533.

Maio, J. J., and Schildkraut, C. L. (1967). *J. Mol. Biol.* **24**: 29.
Marsh, R. E., and Donohue, J. (1967). *Advan. Protein Chem.* **22**: 235.
Marushige, K., and Bonner, J. (1966). *J. Mol. Biol.* **15**: 160.
Marushige, K., and Dixon, G. H. (1969). *Develop. Biol.* **19**: 397.
Marushige, K., and Ozaki, H. (1967). *Develop. Biol.* **16**: 474.
Marushige, K., Brutlag, D., and Bonner, J. (1968). *Biochemistry* **7**: 3149.
Marushige, K., Ling, V., and Dixon, G. H. (1969). *J. Biol. Chem.* **244**: 5953.
Marzluff, W. F., Jr., McCarty, K. S., and Turkington, R. W. (1969). *Biochim. Biophys. Acta* **190**: 517.
Matsuo, K., and Tsuboi, M. (1969). *Biopolymers* **8**: 153.
Mauritzen, C. M., Starbuck, W. C., Sarojo, I. S., Taylor, C. W., and Busch, H. (1967). *J. Biol. Chem.* **242**: 2240.
Meisler, M. H., and Langan, T. A. (1967). *J. Cell Biol.* **35**: 91.
Meisler, M. H., and Langan, T. A. (1969). *J. Biol. Chem.* **244**: 4961.
Millette, R. L., and Trotter, C. D. (1970). *Proc. Nat. Acad. Sci. U.S.* **66**: 701.
Mohberg, J., and Rusch, H. P. (1969). *Arch. Biochem. Biophys.* **134**: 577.
Moudrianakis, E. N. (1969). *Biophys. J.* **9**: 54 Abstr.
Murray, K. (1964). *Biochemistry* **3**: 10.
Murray, K. (1969). *J. Mol. Biol.* **39**: 125.
Murray, K., and Milstein, C. (1967). *Biochem. J.* **105**: 491.
Murray, K., and Peacocke, A. R. (1962). *Biochim. Biophys. Acta* **55**: 935.
Neelin, J. M. (1968). *Can. J. Biochem.* **46**: 241.
Neelin, J. M., and Butler, G. C. (1961). *Can. J. Biochem. Physiol.* **39**: 485.
Nemer, M., and Lindsay, D. T. (1969). *Biochem. Biophys. Res. Commun.* **35**: 156.
Nohara, H., Takahashi, T., and Ogata, K. (1966). *Biochim. Biophys. Acta* **127**: 282.
Nohara, H., Takahashi, T., and Ogata, K. (1968). *Biochim. Biophys. Acta* **154**: 529.
O'Connor, P. J. (1969). *Biochem. Biophys. Res. Commun.* **35**: 805.
Ogawa, Y., Quagliarotti, G., Jordan, J., Taylor, C. W., Starbuck, W. C., and Busch, H. (1969). *J. Biol. Chem.* **244**: 4387.
Ohba, Y. (1966a). *Biochim. Biophys. Acta* **123**: 76.
Ohba, Y. (1966b). *Biochim. Biophys. Acta* **123**: 84.
Ohlenbusch, H. H., Olivera, B. M., Tuan, D., and Davidson, N. (1967). *J. Mol. Biol.* **25**: 299.
Olins, D. E. (1969). *J. Mol. Biol.* **43**: 439.
Olins, D. E., Olins, A. L., and von Hippel, P. H. (1967). *J. Mol. Biol.* **24**: 157.
Olins, D. E., Olins, A. L., and von Hippel, P. H. (1968). *J. Mol. Biol.* **33**: 265.
Ono, T., Terayama, H., Takaku, F., and Nakao, K. (1969). *Biochim. Biophys. Acta* **179**: 214.
Ord, M. G., and Stocken, L. A. (1966a). *Biochem. J.* **98**: 888.
Ord, M. G., and Stocken, L. A. (1966b). *Biochem. J.* **101**: 34P.
Ord, M. G., and Stocken, L. A. (1966c). *In* "The Cell Nucleus, Metabolism and Radiosensitivity" (Radiobiol. Inst. Organ. Health Res., TNO) p. 137. Taylor & Francis, London.
Ord, M. G., and Stocken, L. A. (1967). *Biochem. J.* **103**: 5P.
Ord, M. G., and Stocken, L. A. (1968a). *Biochem. J.* **107**: 403.
Ord, M. G., and Stocken, L. A. (1968b). *Biochem. J.* **109**: 24P.
Ord, M. G., and Stocken, L. A. (1969). *Biochem. J.* **112**: 81.
Orenstein, J. M., and Marsh, W. H. (1968). *Biochem. J.* **109**: 697.
Paik, W. K., and Kim, S. (1967a). *Biochem. Biophys. Res. Commun.* **27**: 479.
Paik, W. K., and Kim, S. (1967b). *Biochem. Res. Commun.* **29**: 14.

Paik, W. K., and Kim, S. (1968). *J. Biol. Chem.* **243**: 2108.
Paik, W. K., and Kim, S. (1969a). *J. Neurochem.* **16**: 1257.
Paik, W. K., and Kim, S. (1969b). *Arch. Biochem. Biophys.* **134**: 632.
Palau, J., and Butler, J. A. V. (1966). *Biochem. J.* **100**: 779.
Panyim, S., and Chalkley, R. (1969a). *Biochemistry* **8**: 3972.
Panyim, S., and Chalkley, R. (1969b). *Biochem. Biophys. Res. Commun.* **37**: 1042.
Panyim, S., Jensen, R. H., and Chalkley, R. (1968). *Biochim. Biophys. Acta* **160**: 252.
Pardon, J. F., Wilkins, M. H. F., and Richards, B. M. (1967). *Nature (London)* **215**: 508.
Patel, G., Howk, R., and Wang, T. Y. (1967). *Nature (London)* **215**: 1488.
Patterson, B. D., and Davies, D. D. (1969). *Biochem. Biophys. Res. Commun.* **34**: 791.
Paul, J., and Gilmour, R. S. (1966). *J. Mol. Biol.* **16**: 242.
Paul, J., and Gilmour, R. S. (1968). *J. Mol. Biol.* **34**: 305.
Pawse, A. R., Ord, M. G., and Stocken, L. A. (1969). *Biochem. J.* **114**: 54P.
Phillips, D. M. P. (1961). *Biochem. J.* **80**: 40P.
Phillips, D. M. P. (1963). *Biochem. J.* **87**: 258.
Phillips, D. M. P. (1965). *Biochem. J.* **97**: 669.
Phillips, D. M. P. (1967). *Biochem. J.* **105**: 46P.
Phillips, D. M. P. (1968). *Biochem. J.* **107**: 135.
Phillips, D. M. P., and Johns, E. W. (1959). *Biochem. J.* **72**: 538.
Phillips, D. M. P., and Simson, P. (1969). *Biochim. Biophys. Acta* **181**: 154.
Pogo, B. G. T., Allfrey, V. G., and Mirsky, A. E. (1966). *Proc. Nat. Acad. Sci. U.S.* **55**: 805.
Pogo, B. G. T., Allfrey, V. G., and Mirsky, A. E. (1967). *J. Cell Biol.* **35**: 477.
Pogo, B. G. T., Pogo, A. O., Allfrey, V. G., and Mirsky, A. E. (1968). *Proc. Nat. Acad. Sci. U.S.* **59**: 1337.
Pogo, B. G. T., Pogo, A. O., and Allfrey, V. G. (1969). *Genetics* **61** (Suppl.): 373.
Prescott, D. M. (1966). *J. Cell Biol.* **31**: 1.
Pyhtila, M. J., and Sherman, F. G. (1968). *J. Gerontol.* **23**: 450.
Raaf, J., and Bonner, J. (1968). *Arch. Biochem. Biophys.* **125**: 567.
Rall, S. C., and Cole, R. D. (1970). Personal communication.
Rasmussen, P. S., Murray, K., and Luck, J. M. (1962). *Biochemistry* **1**: 79.
Reid, B. R., and Cole, R. D. (1964). *Proc. Nat. Acad. Sci. U.S.* **51**: 1044.
Robbins, E., and Borun, T. W. (1967). *Proc. Nat. Acad. Sci. U.S.* **57**: 409.
Sadgopal, A. (1968). PhD. Thesis, Calif. Inst. Technol., Pasadena, California.
Sadgopal, A., and Bonner, J. (1969). *Biochim. Biophys. Acta* **186**: 349.
Sadgopal, A., and Bonner, J. (1970a). *Biochim. Biophys. Acta* **207**: 206.
Sadgopal, A., and Bonner, J. (1970b). *Biochim. Biophys. Acta* **207**: 227.
Sadgopal, A., and Kabat, D. (1969). *Biochim. Biophys. Acta* **190**: 486.
Schwimmer, S. (1965). *Life Sci.* **4**: 1247.
Schwimmer, S., and Aronson, A. (1967). *Biochim. Biophys. Acta* **134**: 59.
Schwimmer, S., and Bonner, J. (1965). *Biochim. Biophys. Acta* **108**: 67.
Seeley, J. H., and Benoiton, N. L. (1969). *Biochem. Biophys. Res. Commun.* **37**: 771.
Seifart, K. H., and Sekeris, C. E. (1969). *Eur. J. Biochem.* **7**: 408.
Sekeris, C. E., Sekeri, K. E., and Gallwitz, D. (1967). *Hoppe-Seyler's Z. Physiol. Chem.* **348**: 1660.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967). *Biochem. Biophys. Res. Commun.* **28**: 815.

Shapiro, J. T., Leng, M., and Felsenfeld, G. (1969). *Biochemistry* **8**: 3219.
Shepherd, G. R., Noland, B. J., and Roberts, C. N. (1970). *Biochim. Biophys. Acta* **199**: 265.
Sheridan, W. F., and Stern, H. (1967). *Exp. Cell Res.* **45**: 323.
Shih, T. Y. (1969). Ph.D. Thesis, California Inst. of Technol. Pasadena, California.
Shih, T. Y., and Bonner, J. (1970a). *J. Mol. Biol.* **48**: 469.
Shih, T. Y., and Bonner, J. (1970b). *J. Mol. Biol.* **50**: 333.
Shirey, T., and Huang, R. C. (1969). *Biochemistry* **8**: 4138.
Skalka, A., Fowler, A. V., and Hurwitz, J. (1966). *J. Biol. Chem.* **241**: 588.
Smart, J. E. (1970). Ph.D. Thesis, Calif. Inst. Technol., Pasadena, California.
Smart, J. E., and Bonner, J. (1970a). *J. Mol. Biol.* Accepted for publication.
Smart, J. E., and Bonner, J. (1970b). *J. Mol. Biol.* Accepted for publication.
Smart, J. E., and Bonner, J. (1970c). *J. Mol. Biol.* Accepted for publication.
Smith, E. L., DeLange, R. J., and Bonner, J. (1970). *Pharmacol. Rev.* **50**: 159.
Sonnenberg, B. P., and Zubay, G. (1965). *Proc. Nat. Acad. Sci. U.S.* **54**: 415.
Spalding, J., Kajiwara, K., and Mueller, G. C. (1966). *Proc. Nat. Acad. Sci. U.S.* **56**: 1535.
Spelsberg, T. C., and Hnilica, L. S. (1969). *Biochim. Biophys. Acta* **195**: 63.
Spelsberg, T. C., Tunkersley, S., and Hnilica, L. S. (1969). *Proc. Nat. Acad. Sci. U.S.* **62**: 1218.
Stellwagen, R. H., and Cole, R. D. (1968). *J. Biol. Chem.* **243**: 4456.
Stellwagen, R. H., and Cole, R. D. (1969a). *J. Biol. Chem.* **244**: 4878.
Stellwagen, R. H., and Cole, R. D. (1969b). *Annu. Rev. Biochem.* **38**: 951.
Stellwagen, R. H., Reid, B. R., and Cole, R. D. (1968). *Biochim. Biophys. Acta* **155**: 581.
Stevely, W. S., and Stocken, L. A. (1966). *Biochem. J.* **100**: 20C.
Stevely, W. S., and Stocken, L. A. (1968a). *Biochem. J.* **109**: 24P.
Stevely, W. S., and Stocken, L. A. (1968b). *Biochem. J.* **110**: 187.
Stevens, F. C., Glazer, A. N., and Smith, E. L. (1967). *J. Biol. Chem.* **242**: 2764.
Stockdale, F. E., and Topper, Y. J. (1966). *Proc. Nat. Acad. Sci. U.S.* **56**: 1283.
Stocken, L. A., and Ord, M. G. (1966). *In* "The Cell Nucleus, Metabolism and Radiosensitivity" (Radiobiol. Inst. Organ. Health Res., TNO), p. 141. Taylor & Francis, London.
Stocken, L. A., and Ord, M. G. (1969). *Biochem. J.* **114**: 51P.
Swingle, K. F., Cole, L. J., and Bailey, J. S. (1967). *Biochim. Biophys. Acta* **149**: 467.
Takai, S., Borun, T. W., Muchmore, J., and Lieberman, I. (1968). *Nature (London)* **219**: 860.
Teng, C.-S., and Hamilton, T. H. (1969). *Proc. Nat. Acad. Sci. U.S.* **63**: 465.
Teraoka, H. (1968). *Plant Cell Physiol.* **9**: 819.
Tidwell, T., Allfrey, V. G., and Mirsky, A. E. (1968). *J. Biol. Chem.* **243**: 707.
Todd, P., and Gronow, M. (1969). *Anal. Biochem.* **28**: 369.
Trevithick, J. R. (1969). *Biochem. Biophys. Res. Commun.* **36**: 728.
Trevithick, J. R., Ingles, C. J., and Dixon, G. H. (1967). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **26**: 603.
Tuan, D. Y. H., and Bonner, J. (1969). *J. Mol. Biol.* **45**: 59.
Turkington, R. W., and Riddle, M. (1969). *J. Biol. Chem.* **244**: 6040.
Umana, R., and Dounce, A. L. (1964). *Exp. Cell Res.* **35**: 277.
Vaughn, J. C. (1968). *J. Histochem. Cytochem.* **16**: 473.
Vescia, A., Capobianco, G., and Morris, H. P. (1967). *Ital. J. Biochem.* **16**: 350.

Vidali, G., and Neelin, J. M. (1968). *Can. J. Biochem.* **46**: 781.
Vidali, G., Gershey, E. L., and Allfrey, V. G. (1968). *J. Biol. Chem.* **243**: 6361.
Vidali, G., Gershey, E. L., and Allfrey, V. G. (1969). *Abstr. Commun. 6th FEBS Meet., Madrid* Abstr. No. 237.
Vorobyev, V. I., Gineitis, A. A., and Vinogradova, I. A. (1969). *Exp. Cell Res.* **57**: 1.
Wang, J. C., Baumgarten, D., and Olivera, B. M. (1968). *Proc. Nat. Acad. Sci. U.S.* **58**: 1852.
Wang, T. Y. (1967). *J. Biol. Chem.* **242**: 1220.
Wang, T. Y. (1968a). *Exp. Cell Res.* **53**: 288.
Wang, T. Y. (1968b). *Arch. Biochem. Biophys.* **127**: 235.
Wang, T. Y. (1969). *Exp. Cell Res.* **57**: 467.
Wang, T. Y., and Johns, E. W. (1968). *Arch. Biochem. Biophys.* **124**: 176.
Weiss, S. B. (1960). *Proc. Nat. Acad. Sci. U.S.* **46**: 1020.
Wilkins, M. H. F. (1956). *Cold Spring Harbor Symp. Quant. Biol.* **21**: 75.
Wood, W. G., Irvin, J. L., and Holbrook, D. J., Jr. (1968). *Biochemistry* **7**: 2256.
Yarbro, J. W. (1967). *Biochim. Biophys. Acta* **145**: 531.
Yasunobu, K. T., Nakashima, T., Higa, H., Matsubara, H., and Benson, A. (1963). *Biochim. Biophys. Acta* **78**: 791.
Zubay, G., and Doty, P. (1959). *J. Mol. Biol.* **1**: 1.

MOLECULAR MECHANISMS OF CHROMOSOME BREAKAGE AND REJOINING

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I. Introduction

Ten years ago, in a review article on a subject similar to the present one ("Biochemical Aspects of Chromosome Breakage"), I concluded with the rather pessimistic prediction that the problem of chromosome breakage could not be solved by biochemical methods. Although the foundation was already laid for the remarkable success of molecular

biology witnessed during the last decade, and several fundamental discoveries had already been made, the new ideas had not yet penetrated the field of chromosome science.

Today the situation is different. The time seems not very far ahead when chromosome structure and the mechanisms of chromosome breakage and rejoining will be explained by results obtained in the fields of molecular genetics and ultrastructure research. A statement by DuPraw (1969) points the general direction: "While it is far from certain at present that the principles of chromosomal organization extracted so neatly from bacteria and viruses can be administered unchanged to other creatures, what is certain is that the science of chromosomes is experiencing a genuine transformation, in which the properties of DNA are the key to the change." There is ample evidence that DNA is the principal target in cellular inactivation and in mutation induction by physical and chemical agents (Haynes, 1966; Hanawalt, 1968). The data collected in this review article also suggest that DNA is the chemical species principally involved in the formation of chromosomal aberrations by such agents.

II. Types of Aberrations and the Mechanics of Aberration Formation

A. Types of Aberrations Observed with the Light Microscope

It is customary to distinguish three main categories of chromosomal aberrations: subchromatid-, chromatid-, and chromosome-type aberrations (to be defined later in this section). Depending on their morphological appearance during the stages of active division, these aberrations are usually scored either as breaks or as exchanges.

A type of aberration which does not readily fit into either of these categories is the "gap" or achromatic lesion. Not even the subchromatid exchanges have given rise to so much controversy in the past as the gaps. In contrast to a break, a gap is not supposed to give rise to a free fragment in anaphase because the part of the chromatid on the distal side of the gap is still connected with the rest of the chromatid. Nevertheless, the connecting material frequently cannot be visualized by the usual staining methods. Gaps usually extend through the whole chromatid, but they may also involve only part of the diameter of the chromatid. At a particular locus, gaps may occur in one of the sister chromatids (chromatid gap) or in both (isochromatid gap). At metaphase it can be very difficult to distinguish between gaps and breaks

(Fig. 1a,b,c), and it is this difficulty which has caused most of the controversy.

Subchromatid aberrations are also controversial. In this type of aberration, the unit of breakage appears to be smaller than a single chromatid (Fig. 7). Subchromatid exchanges arise when cells are irradiated with ionizing radiation in prophase or are treated with chemicals such as coumarin (Östergren, 1948) or methylated oxypurines (Kihlman, 1952).

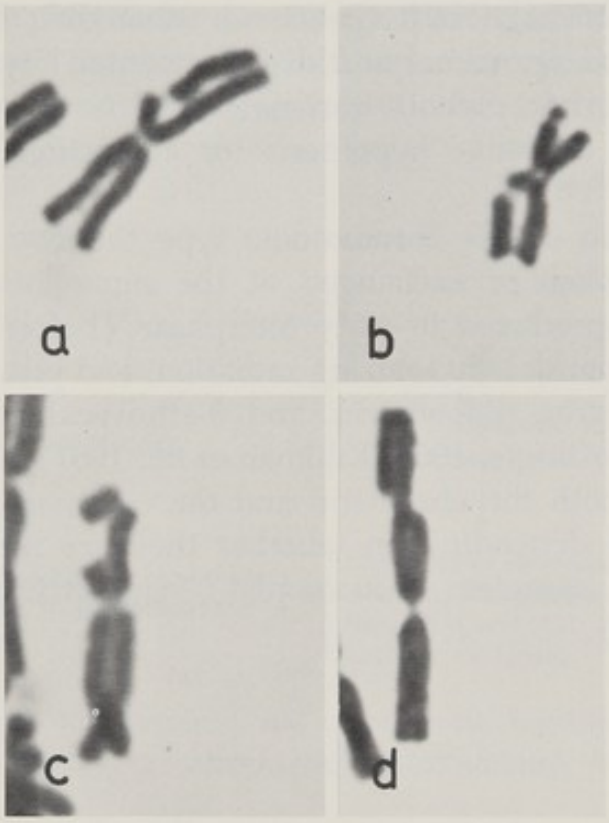

FIG. 1. Apparent and real chromatid discontinuities in metaphase chromosomes of *Allium*. (a) and (b) would be scored as gaps by most authors, (c) and (d) as chromatid breaks. [a, c, d from Kihlman (1970b). Reprinted by permission of Plenum Publishing Corporation.]

At anaphase, subchromatid exchanges give rise to characteristic side-arm bridges or "pseudo-chiasmata" (Levan and Tjio, 1948). The controversy concerns the true nature of these subchromatid changes. Possibilities to be considered are (1) that exchanges occur between half-chromatids (Crouse, 1954; Sax and King, 1955; Wilson *et al.*, 1959), (2) that they involve a varying number of subchromatid strands (LaCour and Rutishauser, 1954), and (3) that apparent subchromatid exchanges are masked chromatid exchanges (Östergren and Wakonig, 1954; Kihlman and Hartley, 1967a; Kihlman, 1970a). In Section IV,B,1, evidence will be presented indicating that the third alternative is the most likely one.

In an aberration of the chromatid type, the unit of breakage is the single chromatid. Chromatid-type breaks and exchanges are formed in middle and late interphase. Many chemical mutagens produce aberrations which are exclusively of the chromatid type. At a particular locus, breaks may occur in one of the sister chromatids (chromatid break) or in both (isochromatid break). Depending on the completeness of union between the sister chromatids at the point of breakage, it is customary to distinguish between isochromatid breaks of the SU (proximal and distal sister-union), NUp (proximal nonunion), NUd (distal nonunion), and NUpd (proximal and distal nonunion) types. A new interpretation of the origin of both chromatid and isochromatid breaks was given in Revell's exchange hypothesis for aberration formation (to be described).

In an aberration of the chromosome type the two sister chromatids appear to be broken or exchanged at the same locus. This category of aberrations is produced in early interphase (before the chromosome is split into chromatids) by ionizing radiation, and possibly by chemicals such as streptonigrin, phleomycin, and 8-ethoxycaffeine (Kihlman and Odmark, 1965; Mattingly, 1967; Kihlman *et al.*, 1967).

Exchanges of both the chromatid and the chromosome type may further be classified depending on whether they are interchromosome or intrachromosome, complete or incomplete, symmetrical or asymmetrical (see Evans, 1962).

B. Hypotheses of Aberration Formation

According to the general or breakage-rejoining hypothesis (Sax, 1939, 1940, 1941), the primary event caused by a chromosome-breaking agent is a chromatid or chromosome break. The chromosome configuration observed at mitosis depends on what happens to these breaks. If the ends at the point of breakage rejoin to restore the original configuration (restitution), the affected chromosome will be normal at mitosis, and according to the hypothesis most breaks undergo restitution. However, the break may also remain open, in which case at mitosis either a chromosome or a chromatid break is observed, depending on the stage of interphase when the break was induced. A final possibility is that the ends may rejoin with the ends from another break, provided that the two breaks are close enough in space and time (illegitimate fusion); the results of such illegitimate fusion are the unions of sister chromatids in an isochromatid break or the various types of exchange. Figure 2 summarizes the various consequences of such breaks.

According to the exchange hypothesis proposed by Revell (1955,

1959), the primary event in aberration formation is not a break but a local region of instability in the chromosomes (lesion). The lesion decays with time, meaning that it either reverts to normal or that it is transformed to a state which, although abnormal, is incapable of taking part in exchange formation. When two lesions are close enough in space and time, they may enter another, more stable stage which is called "exchange initiation" by Revell. In the exchange initiation stage, which can be of considerable duration, no genetic changes have yet occurred, but this stage is the prelude for a mechanical exchange process.

Revell's hypothesis implies that all types of chromatid aberrations arise as a result of an exchange process. The isochromatid break of

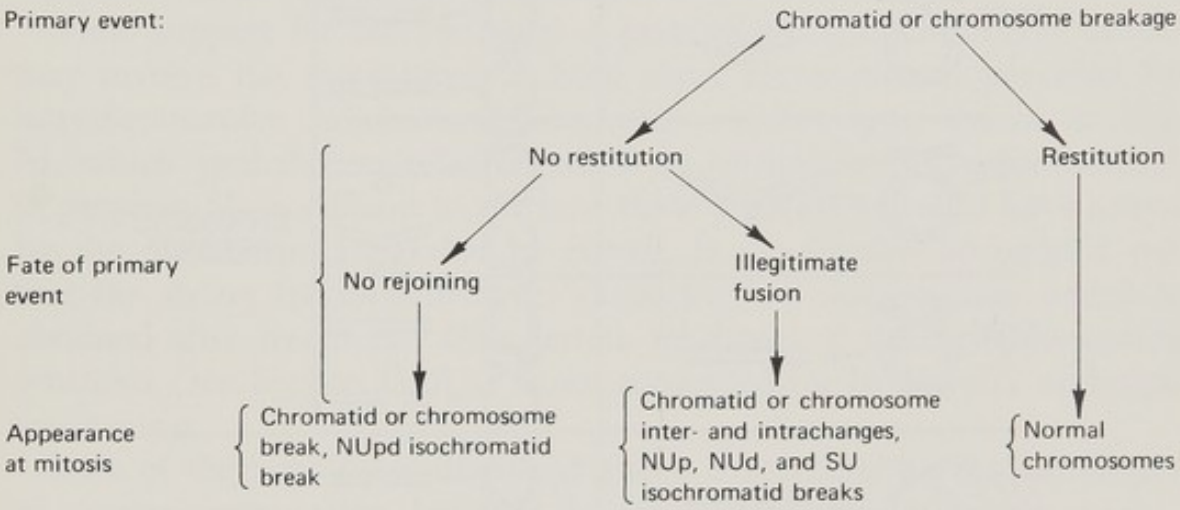

FIG. 2. Consequences of chromatid and chromosome breakage according to the breakage-rejoining hypothesis.

the breakage-rejoining hypothesis is, in the exchange hypothesis, one of four possible types of chromatid intrachange (Fig. 3), and does not involve the same locus in the two sister chromatids. Chromatid breaks arise as a result of incomplete exchange and are a comparatively rare type of aberration. According to Revell, the explanation for the high frequencies of chromatid breaks observed in irradiated cells by previous workers would be that gaps have erroneously been scored as breaks.

Experimental data published by Revell (1959) were in good agreement with the exchange hypothesis, and his results were soon confirmed by other English workers (e.g., Evans and Neary, 1958; Evans, 1962; Neary and Savage, 1966). Nevertheless, for many years the ideas expressed in the hypothesis were strongly resisted by most workers in the field (e.g., see Wolff, 1963). More recently there has been a definite change in the current attitude toward the exchange hypothesis; for example, Brewen and Brock (1968) obtained evidence that chromosome-type deletions originate from two-hit events, and they suggested that

these aberrations arise by incomplete exchange in accordance with Revell's exchange hypothesis, rather than by a mechanism of breakage and rejoining. Since Revell has worked out the exchange hypothesis only for chromatid-type aberrations, the results of Brewen and Brock

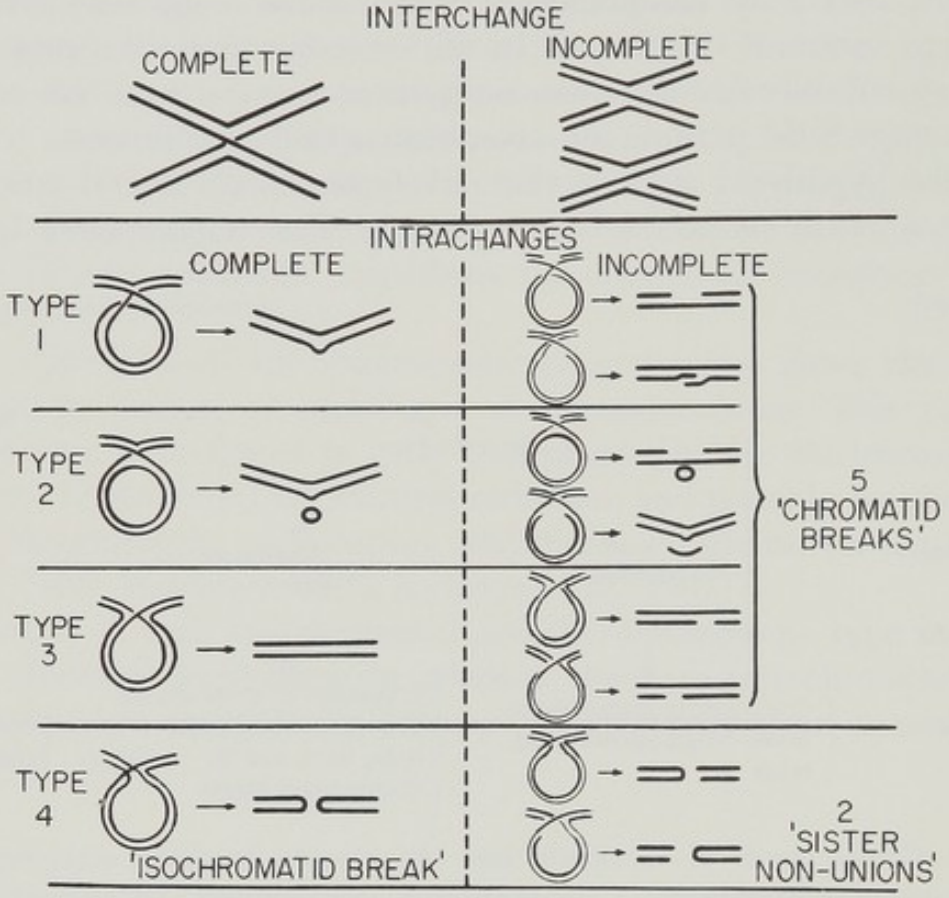

FIG. 3. Diagram of Revell's exchange hypothesis. The complete forms of exchange are shown on the left and the incomplete forms on the right. Note that there should be five "chromatid breaks" for every two incomplete "isochromatid breaks." (From Evans and Scott, 1969.)

have special significance. From a study on chromatid deletions in Chinese hamster cells, Heddle and Bodycote (1970) have concluded that these aberrations are a mixture of unrejoined single breaks and incomplete exchanges between sister chromatids.

During the last few years additional studies by English workers have provided further support for Revell's hypothesis (Scott and Evans, 1967; Savage *et al.*, 1968; Savage, 1968; Evans and Scott, 1969). However, at one point the results do not conform with the exchange hypothesis. Although it was expected that the ratio between the total number of chromatid discontinuities (chromatid breaks) and the total number of incomplete (sister nonunion) isochromatid breaks should be 5:2, for high

LET (linear energy transfer) particles, Savage *et al.* (1968) and Savage (1968) obtained ratios considerably lower than 2.5. Similarly in the X-ray experiments of Scott and Evans (1967), the ratios were also much lower than expected, particularly when the cells were exposed during the period of interphase DNA synthesis. After treatments with nitrogen mustard, Evans and Scott (1969) obtained ratios which were very similar to those found in cells exposed to X-rays during DNA synthesis. Evans and Scott (1969) concluded that "a proportion of the isochromatid aberrations induced by the chemical agents, and also by X-rays in S, *are not the result of an exchange between two loci, one on each of two sister chromatids, as envisaged by Revell, but are produced as a result of misreplication at a single locus*" (Evans and Scott, 1969).

Some support for the idea that a percentage of isochromatid breaks may involve the same locus in both sister chromatids is provided by intrachromosome subchromatid exchanges of the type shown in Fig. 7d, which probably can be regarded as isochromatid breaks induced in prophase. It is difficult to see how these aberrations could have arisen by the mechanism suggested by Revell. It should also be pointed out that the strong fragmentation or "shattering" of chromosomes which is obtained after treatments with certain inhibitors of deoxyribonucleotide synthesis (see Section IV,B,5) is not accounted for by Revell's exchange mechanism.

Both of the hypotheses discussed above concentrate on the *mechanics* of aberration formation, but they do not deal with the *molecular* mechanisms involved. In order to be able to discuss the biochemical aspects of chromosome breakage and rejoining, it will be necessary to survey some recent findings and ideas about chromosome structure and synthesis.

III. The Chemistry, Ultrastructure, and Replication of Chromosomes

A. Chemical Components of Chromosomes

The fact that the chromosomes of eukaryotic organisms are composed mainly of nucleic acid and protein has been known for decades. During the last few years accurate analyses of the relative proportions of these components in isolated metaphase chromosomes have been published (e.g., Salzman *et al.*, 1966; Huberman and Attardi, 1966; Maio and Schildkraut, 1967); it was found that mammalian metaphase chromosomes contain 66–74% protein, 15–20% DNA and 10–14% RNA. Somewhat less than half of the protein is of the histone type. The RNA in isolated

chromosomes consists mainly of ribosomal RNA and should be regarded as a chromosomal product, rather than as a chromosomal constituent. In addition, the presence of lipid material in chromosomes has been suggested by cytochemical studies (Chayen *et al.*, 1957; LaCour *et al.*, 1958; Gahan, 1965).

During the various phases of the mitotic cycle, the amount of DNA per chromatid appears to be relatively constant, whereas the amounts of RNA and protein vary. Thus, the amounts of protein and ribosomal RNA in relation to DNA are considerably higher in metaphase chromosomes than in interphase chromatin (for references, see DuPraw, 1970). Apparently, mitotic chromosomes are able to pick up ribosomes from the disintegrating nucleolus and from the cytoplasm.

Although the chemical composition of chromosomes is well known, the question of how the various components are put together is still under investigation. Callan and MacGregor (1958; also MacGregor and Callan, 1962) studied the effects of various enzymes on the structure of lampbrush chromosomes isolated from amphibian oocytes. They found that the axis of the loops in the lampbrush chromosomes is broken by DNase but not by trypsin, pepsin, or RNase, and they concluded that uninterrupted fibers of DNA run throughout the length of these chromosomes. The studies of Gall (1963a) on the kinetics of DNase digestion of lampbrush chromosomes suggested that there are two subunits of DNA (one DNA double helix) in the loops, corresponding to one chromatid, and four subunits (a pair of DNA double helices) along the main axis, corresponding to two chromatids. Thus, these studies suggest that the linear continuity of lampbrush chromosomes is dependent on DNA and that the structural "backbone" in a chromatid probably consists of a single DNA double helix. More information on this point has been obtained in studies of chromosome ultrastructure.

B. Chromosome Ultrastructure

Until the surface-spreading technique of Kleinschmidt *et al.* (1962) was modified by Gall (1963b) for the study of chromosome ultrastructure, the electron microscope was not very useful as a tool in chromosome research. However, with the aid of the spreading technique and the critical point drying method of Anderson (1950), important information has been obtained in electron microscopic studies.

Thus, it has been demonstrated that interphase nuclei from various tissues of animals and plants contain chromatin fibers that are 200–250

Å thick (e.g., DuPraw, 1965a,b; Gall, 1966; Ris, 1967; Wolfe, 1968). In the presence of chelating agents, such as EDTA or sodium citrate, the 250 Å fibers are transformed into fibrils 80–100 Å thick (Ris, 1967). According to Ris (1967), the 250 Å fibers are formed by folding of 100 Å fibers and the 100 Å fiber should therefore be regarded as the basic unit of organization of the DNA–histone complex in chromatin. The recent studies of DuPraw and Bahr (1969) have also revealed the presence of two types of fibers in chromatin, i.e., "type A" and "type B" fibers, the diameters of which are 50–110 Å and 200–500 Å, respectively. When studied by quantitative electron microscopy, the type A fiber proves to be intermediate, not only in diameter, but also in specific gravity and dry mass per micron, between extended DNA molecules and type B fibers. According to DuPraw and Bahr (1969), the type A fibers "appear to be first-order DNA-protein supercoils which undergo second-order supercoiling to generate (coiled coil) type B fibers."

The spreading technique has also been used successfully to prepare metaphase chromosomes as whole mounts for electron microscopy (e.g., DuPraw, 1965a,b, 1966, 1968, 1970; Gall, 1966; Ris, 1967). These metaphase chromosomes contain the same type of fibers as interphase nuclei. No central core or backbone has been seen in metaphase chromatids, nor any outer limiting membrane (e.g., DuPraw, 1968; Comings and Okada, 1969). The chromosomes do not contain any half or quarter chromatids (DuPraw, 1966, 1970; Comings and Okada, 1969). These studies suggest that metaphase chromosomes consist entirely of tightly packed chromatin fibers and that they are formed from interphase fibers by combined transverse and longitudinal folding (Fig. 4). Superimposed on the cylindrical folded-fiber unit, a pattern of regular quaternary coiling (resolvable in the light microscope) frequently occurs (e.g., DuPraw, 1965b, 1966; Abuelo and Moore, 1969; Frederic, 1969; Schwarzacher and Schnedl, 1969). According to DuPraw (1965b, 1966), who proposed the folded-fiber model of chromosome structure, each metaphase chromatid contains one single folded fiber and is connected with its sister chromatid by unreplicated portions of the fiber, especially near the centromeric region.

Enzyme digestion studies have revealed that each chromatin fiber consists of a trypsin sensitive portion and a 25–50 Å trypsin resistant axis. The axis stains with uranyl acetate and is sensitive to DNase (DuPraw, 1965a,b; Ris, 1967; Abuelo and Moore, 1969). These findings strongly suggest that the chromatin fiber consists of a single DNA double helix with attached protein. If the chromatin fiber contains one DNA double helix and a chromatid contains one fiber, then there should be only one DNA double helix per chromatid; this conclusion of DuPraw

is compatible with the results obtained by Gall (1963a) in his studies on the kinetics of DNase action on lampbrush chromosomes (see Section III,A).

By using the spreading technique for the preparation of lampbrush chromosomes for electron microscopy, Miller (1965) was able to show that the minimum diameter of DNase sensitive lateral loop axes (one chromatid) is 20–30 Å, or the same as the diameter of a single DNA double helix. Other more recent electron microscopic studies have also yielded results which fit the one-DNA-helix-per-chromatid model of chromosome structure (Hsu, 1968; Comings and Okada, 1969; Schwarzacher and Schnedl, 1969). This model is also compatible with the results of autoradiographic studies on the replication and segregation of DNA in chromosomes (see Section III,C) and with recombination, mutation, and linkage data.

If this model is correct, a question arises whether the DNA exists as a single, uninterrupted molecule from one end of the unit fiber to the other, or whether a series of DNA molecules are tandemly joined end to end, perhaps by non-DNA material. It is possible to estimate the DNA content of a chromatid with reasonable accuracy. On the basis of the figure thus obtained, the total length of a single DNA molecule containing all this DNA can be calculated. It appears that the largest chromosome in a human cell would contain a DNA helix 7.3 cm long and the smallest a DNA molecule 1.4 cm long (DuPraw, 1968). By using an autoradiographic technique, Huberman and Riggs (1966) were able to find molecules as long as 1.8 mm in cells of the Chinese hamster. Sasaki and Norman (1966) used the same method to demonstrate DNA molecules which were between 1 and 2.2 cm long in human cells. To find out if the long DNA filaments observed in hamster cells represent several smaller molecules linked together by protein, Riggs and Huberman (1967) digested the fibers with as much as 1 mg/l Pronase for 6 hours. Since the treatment had no effect on the length of the filaments, the authors concluded that "if any linkers are present, they must be extremely resistant to Pronase."

Studies such as these suggest that each chromatid contains one continuous DNA molecule. On the other hand, it has been shown that chromosomal DNA contains many tandemly joined replicating units or "replicons" (e.g., Painter *et al.*, 1966; Huberman and Riggs, 1968; Taylor,

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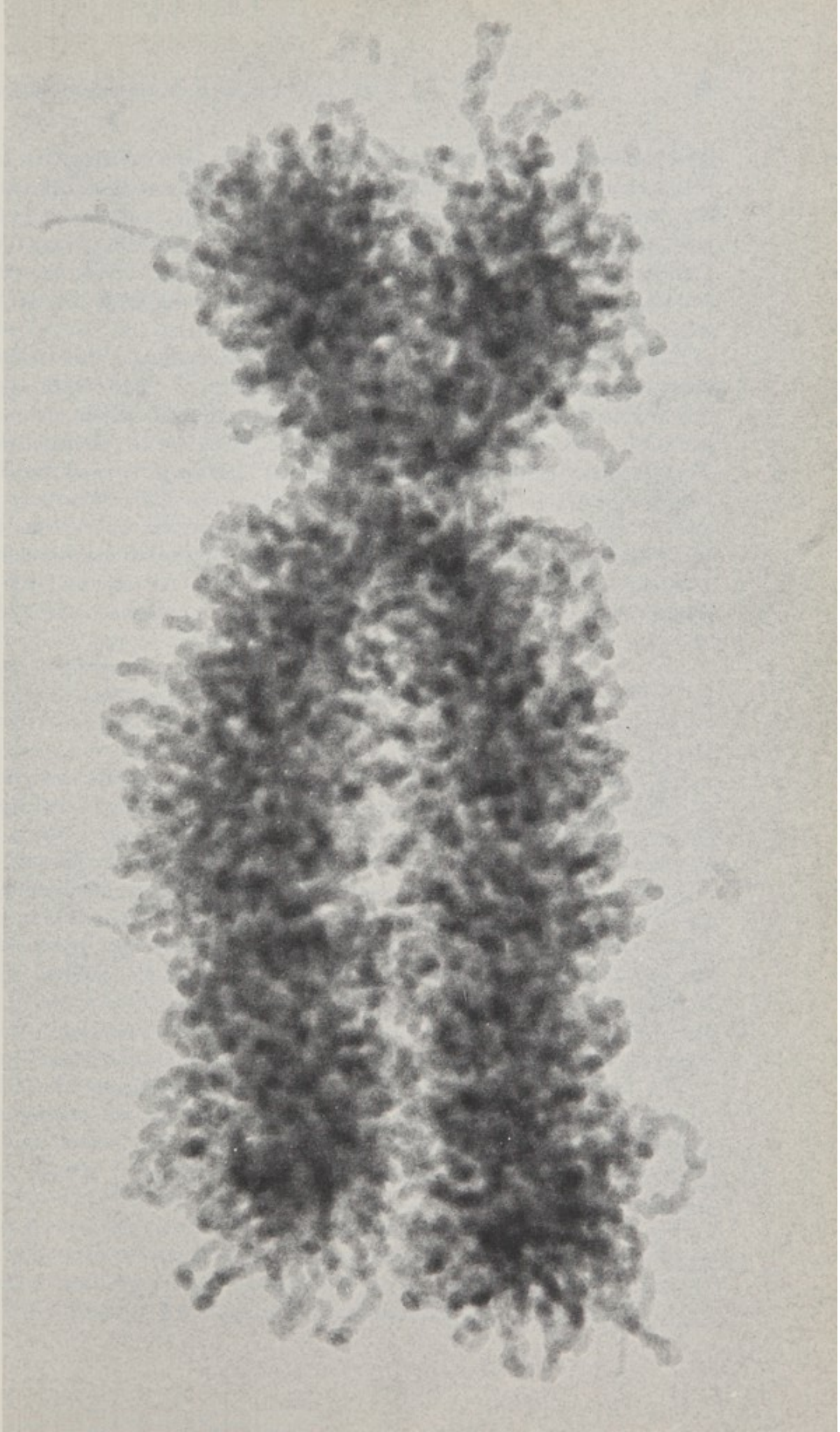
FIG. 4. Electron micrograph of a human chromosome 12 from a lymphocyte culture, arrested in metaphase by colchicine. Prepared as a whole mount according to the surface-spreading and critical point drying techniques. [From DuPraw (1970). *DNA and Chromosomes.* Holt, Rinehart & Winston, Inc.]



1968), which in the Chinese hamster probably are less than 30 μ long (Huberman and Riggs, 1968). In other experiments evidence has been obtained suggesting that chromosomal DNA under certain conditions (e.g., high pH) may fall apart into subunits about 100 μ long (e.g., Taylor, 1969; Humphrey *et al.*, 1968; Corry and Cole, 1968). Nevertheless, no conclusive evidence for the existence of non-DNA linkers between the subunits has been presented.

The findings described so far in this section indicate that a mitotic chromosome is formed by coiling and folding of fiber consisting of a long, single DNA molecule associated with protein. Each chromatid probably contains one such fiber, and it is the DNA of the fiber which is responsible for the structural integrity of both the fiber and the chromatid. From this it can be concluded that the DNA moiety of the chromosome is also likely to be the molecular species primarily involved in chromosome breakage and rejoining. Before we can return to the main subject of this article, i.e., chromosome breakage and rejoining, it will be necessary to deal briefly with the replication of chromosomal DNA.

C. Normal Replication of DNA and Other Chromosomal Constituents during Interphase

Cytophotometric studies have shown that in dividing cells, the amount of DNA per nucleus doubles during interphase and is equally distributed between the two daughter nuclei at anaphase. However, DNA is not synthesized during the entire interphase period; at the beginning and the end of interphase there are normally periods when no DNA synthesis can be detected by autoradiographic methods. That DNA synthesis takes place only during a limited period of interphase was first demonstrated by Howard and Pelc (1953) with the autoradiographic method; these authors also coined the symbol "S" for the DNA synthesis period, and the symbols "G_1" and "G_2" (gap 1 and gap 2) for the interphase periods between telophase and S and between S and prophase, respectively.

The periods G_1, S, and G_2 are characteristic of the various stages of interphase in dividing cells, but they do not exist in the resting stage of differentiated and/or mitotically inactive cells. The symbol "G_0" has been suggested for this stage, which is not part of a mitotic cycle (Lajtha, 1963).

The time of synthesis of another main constituent of chromosomes, the histone proteins, has also been thoroughly investigated, and it has been found that the periods of DNA and histone synthesis coincide closely (e.g., Alfert, 1955; McLeish, 1959; Bloch *et al.*, 1967; Robbins

and Borun, 1967; Gurley and Hardin, 1968; Das and Alfert, 1968). For the nonhistone proteins of chromosomes the evidence is less conclusive, but suggests that synthesis of these proteins is less closely coupled with DNA synthesis than is the synthesis of histones (e.g., Mueller, 1969; Jockusch *et al.*, 1970).

The old problem of whether one sister chromatid is composed of entirely new material while the other contains parental material, or whether both chromatids are half old and half new was solved by Taylor *et al.* (1957) in their now classic study on the replication and segregation of chromosomal DNA in root tip cells of *Vicia faba.* In this autoradiographic study, tritiated thymidine (^{3}H-TdR) was used for the first time to label newly synthesized chromosomal DNA, and the success of the experiment was largely a consequence of this choice of DNA precursor. During the last decade, the method of using ^{3}H-TdR for DNA labeling in autoradiographic experiments has proved to be extremely useful and is now one of the most important and commonly used methods of chromosome analysis. Results obtained by this method have greatly influenced current thinking about the structural organization and synthesis of chromosomes.

Taylor *et al.* (1957) found that the ^{3}H-TdR incorporated into the DNA of chromosomes is part of a structural unit that remains intact during subsequent chromosome duplications. The results further indicate that the chromosome in G_1 consists of two subunits or strands. When duplication starts, these subunits separate and each synthesizes a new subunit. As a result, each chromatid receives one "old" (parental) and one "new" subunit. Subsequently, similar *semiconservative* chromosome duplication patterns have been observed in a large variety of both plant and animal materials by Taylor, as well as by other workers. Although other interpretations are also possible, the observation that the chromosome subunits replicate and segregate semiconservatively, like the two complementary strands of a DNA molecule, suggests that the chromosome subunits are identical with the DNA strands and supports the idea that the chromatid consists essentially of a single DNA double helix with associated protein.

A further important observation obtained in autoradiographic experiments is that different chromosomes of the same set, and even different parts of the same chromosome, may duplicate at different stages of the S period. This phenomenon is particularly striking for sex chromatin; thus, Lima-de-Faria (1959) found that the heterochromatic block formed by the sex chromosomes in the grasshopper *Melanoplus differentialis* synthesizes DNA later than the euchromatic autosomes. Late replication of sex chromatin has also been observed in mammalian cells in tissue

culture (e.g., Taylor, 1960; Grumbach *et al.*, 1963). In root tips of *Vicia faba*, Evans (1964) found that the heterochromatic parts of the chromosomes synthesize their new DNA later than the euchromatic parts, and he concluded that late replication is characteristic of DNA in positive heteropycnotic regions, whether they are in autosomes or in sex chromatin.

It has already been mentioned (Section III,B) that each chromosome contains many replicating units or replicons. In each of these replicons DNA is replicated at a fork-like growing point (e.g., Huberman and

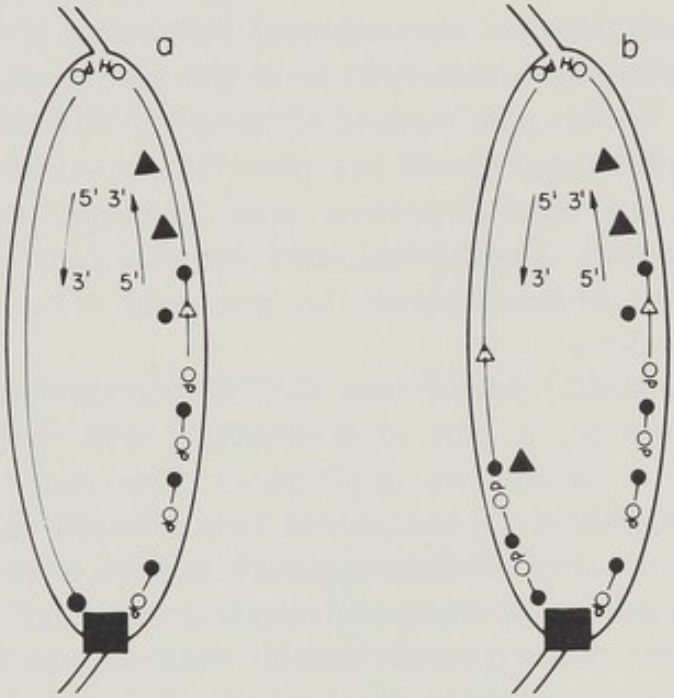


FIG. 5. Model for DNA synthesis by discontinuous growth. At regular intervals at the growing point new chains would be initiated by DNA polymerase; these chains elongate in the 5′ to 3′ direction. Later the newly replicated chains are sealed by DNA ligase into longer chains. ■ growing point; ● DNA polymerase; △▲ DNA ligase. (From Yudelevich *et al.*, 1968.)

Riggs, 1968; Coleman and Okada, 1968). According to Taylor (1969), replication is initiated at each replicon by the breakage of the parental or template strands.

Density gradient studies combined with pulse labeling of newly synthesized DNA have recently provided evidence that DNA is first synthesized in small pieces which are quickly joined to larger segments. Later, these segments are linked together into the final DNA product (Taylor and Straubing, 1968; Painter, 1968a; Schandl and Taylor, 1969; Painter and Schaefer, 1969; Kidwell and Mueller, 1969; Habener *et al.*, 1969).

Results obtained by Painter and his collaborators suggest that the small pieces of newly synthesized DNA, which may contain less than 2000 nucleotides, are partially single stranded (Painter, 1968a; Painter and Schaefer, 1969). Similar observations have been made in bacteria and phages (Okazaki *et al.*, 1968; Sugimoto *et al.*, 1968; Yudelevich *et al.*, 1968; Oishi, 1968a,b,c). These findings are very significant because they show how replication can proceed on both template strands of DNA simultaneously, in spite of the fact that DNA polymerase *in vitro* is capable of carrying out DNA replication only by the incorporation of deoxyribonucleotides in the 5′ to 3′ direction. Okazaki and his collaborators have suggested that DNA replication occurs in a discontinuous manner and in the 5′ to 3′ direction on both strands of DNA, and that the short chains thus formed are joined together by polynucleotide ligase (Okazaki *et al.*, 1968). This hypothesis is supported by the finding that there is an accumulation of newly synthesized short chains of DNA in *E. coli* infected with ligase defective T4 phages (Sugimoto *et al.*, 1968). It is further supported by the observation that the apparent inhibition of DNA synthesis in NAD (nicotinamide-adenine dinucleotide) deficient *E. coli* is due to suppression of the cells' ligase activity (Nozawa and Mizuno, 1969), because NAD functions as a cofactor in the ligase reaction in *E. coli* (Zimmerman *et al.*, 1967; Olivera and Lehman, 1967). Regardless whether Okazaki's hypothesis is correct or not, the results suggest that the DNA ligase reaction is the final step, not only in repair replication, but also in normal, semiconservative DNA synthesis.

D. Repair Replication and Unscheduled DNA Synthesis

Direct evidence for repair replication in UV-irradiated bacteria was provided by Pettijohn and Hanawalt (1964). This type of synthesis is characterized by the excision and replacement of damaged nucleotides in DNA, and since parental material is removed and replaced in this process, it is also called *nonconservative* DNA replication. There is no net increase in amount of DNA in nonconservative replication.

Repair of UV damage to DNA is assumed to involve the following steps: (1) An endonuclease (incision enzyme) recognizes the UV-induced distortion of the double helix and makes a single-strand break close to the primary damage (pyrimidine dimer; see Section IV,A,1); (2) an exonuclease (excision enzyme) removes a single-stranded fragment containing the damaged nucleotides; (3) DNA polymerase inserts new nucleotides into the gap complementary to those of the intact opposite strand; and (4) polynucleotide ligase closes the final

phosphodiester link (for references, see Hanawalt, 1968). Recent findings suggest that DNA polymerase can catalyze not only the replacement but also the excision step (Kelly *et al.*, 1969).

Subsequently, it has been demonstrated that repair replication occurs also after X-irradiation (Emmerson and Howard-Flanders, 1965; Painter and Cleaver, 1967) and after treatment with alkylating agents (Hanawalt and Haynes, 1965; Reiter *et al.*, 1967; Roberts *et al.*, 1968; Strauss *et al.*, 1968). Because ionizing radiation and certain alkylating agents (e.g., methyl methane sulfonate) are capable of inducing DNA strand breaks (Strauss *et al.*, 1968), the recognition and incision steps may not always be required in these cases. It is also likely that the endonuclease which attacks methylated DNA is different from the enzyme capable of attacking UV-damaged DNA (Strauss and Robbins, 1968).

In mammalian cells, nonconservative DNA replication has been demonstrated to occur after moderate to high doses of UV (100 to 800 ergs/mm^2; Cleaver and Painter, 1968) and high doses of X-rays (10^5 R; Painter and Cleaver, 1967). Low levels of repair replication have been observed to occur in "undamaged" (unirradiated) human diploid cells. In contrast to the DNA that underwent repair replication after irradiation with 100 ergs/mm^2 UV, nearly all the "repaired" DNA in unirradiated cells was subsequently able to participate in normal semiconservative replication (Rasmussen *et al.*, 1970). Extensive post-irradiation DNA degradation of the kind that is known from bacterial systems (Pettijohn and Hanawalt, 1964; Aoki *et al.*, 1966; Boyce, 1966) does not occur in mammalian cells (Cleaver, 1968a; Painter, 1968b; Hill, 1969).

When in autoradiographic experiments cells incorporate radioactive DNA precursors (usually ^{3}H-TdR) during G_1 and G_2, as well as during S, the process is called *unscheduled* DNA synthesis. Non-S-phase incorporation of ^{3}H-TdR into DNA of UV-irradiated mammalian cells was first reported by Rasmussen and Painter (1964). Subsequently, the phenomenon has been observed after UV- and X-irradiation in many different types of mammalian cells (Rasmussen and Painter, 1966; Hill, 1967; Djordjevic and Tolmach, 1967; Evans and Norman, 1968), but so far it has not been detected in plant cells. Unscheduled DNA synthesis increases with dose (Rasmussen and Painter, 1966), but unlike repair replication it can be observed also after low dosages of UV (10–25 ergs/mm^2) (Rasmussen and Painter, 1966; Evans and Norman, 1968). Furthermore, the phenomenon is not restricted to irradiated cells; for example, unscheduled DNA synthesis has been observed in Chinese hamster cells after treatment with monofunctional alkylating agents (Hahn *et al.*, 1968), and recently spontaneous incorporation of ^{3}H-TdR

has been reported to occur during G_1 in HeLa cells (Djordjevic *et al.*, 1969).

Rasmussen and Painter (1966) were able to show that part of the synthesis which occurred in HeLa cells after large doses of UV was not semiconservative DNA replication. Many data indicate that unscheduled DNA synthesis and repair replication "reflect the same primary process" (Painter and Cleaver, 1969). The presence of BUdR (5-bromodeoxyuridine) in the DNA of mammalian cells increases unscheduled DNA synthesis after UV irradiation, but it also decreases survival. Thus, the amount of successful repair is not necessarily proportional to the extent of repair replication (Painter and Cleaver, 1969). Recently, Rasmussen *et al.* (1970) have demonstrated that at high levels of damage, the repair system is not capable of producing functional DNA. The authors therefore believe "that the biologically significant function of mammalian DNA repair replication is to repair low levels of DNA damage, e.g., that occurring as a result of 'normal wear and tear.'"

IV. Molecular Mechanisms of Aberration Formation in Chromosomes

A. Responses of DNA to Treatment with Chromosome-Breaking Agents

In the previous section, results were presented which suggest that a single chromatid contains a single, long, folded fiber consisting of a DNA double helix with attached protein. The evidence further suggests that the DNA component is responsible for the structural integrity of the fiber, as well as of the chromatid, and therefore is likely to be the molecular species involved in chromosome breakage and rejoining. It seems appropriate therefore to begin this section with a survey of some *in vivo* responses of DNA molecules to chromosome-breaking agents.

1. *Radiation*

The photoproducts of UV radiation have proved favorable for studies on structural defects produced by mutagens in DNA. Of these photoproducts, pyrimidine dimers are the most frequently occurring. Pyrimidine dimers are formed between adjacent pyrimidine bases in the same single strand, and they can be converted back to monomeric pyrimidines by irradiation with visible light of short wavelength (photoreactivation).

UV-induced pyrimidine dimers may also be removed from DNA and replaced by the mechanism of repair replication (Section III,D).

The presence of pyrimidine dimers in DNA interferes with DNA replication. When a dimer-containing DNA is replicated, the newly synthesized strands are of low molecular weight, evidently because they contain gaps at a spacing approximately equal to that between the pyrimidine dimers (Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968). The data also suggest that these gaps are directly opposite the dimers. Another biologically important effect of UV is probably DNA-protein cross-linking (Smith, 1966). Chain breakage by UV appears to occur too infrequently at low dosages to be of biological importance (Smith, 1966). However, in cells which have their DNA thymine partially replaced with 5-bromouracil, chain breakage becomes an important effect of UV light (e.g., Hutchinson and Koehrlin, 1967).

By contrast, the damage produced by ionizing radiations frequently involves chain breakage. Among the changes produced in DNA by ionizing radiation both *in vivo* and *in vitro*, Kanazir (1969) lists base destruction together with both single- and double-strand breaks. These breaks may be produced by direct hits of the ionizing particle or indirectly by the attack of peroxides and radicals formed from water or organic compounds as a result of the irradiation. In most materials, about ten single-strand breaks are produced for every double-strand break (Freifelder, 1966; Corry and Cole, 1968; Veatch and Okada, 1969). It has been estimated that in the presence of oxygen, 50 eV is required to produce one single-strand break (Dean *et al.*, 1969) and 600 eV to produce a double-strand break. The single-strand breaks are repairable (Freifelder, 1966; Lett *et al.*, 1967; Humphrey *et al.*, 1968). There is evidence which suggests that adenosine triphosphate (ATP) is required for the rejoining of X-ray-induced breaks in the DNA of mammalian cells (Matsudaira *et al.*, 1969, 1970). It is perhaps significant that the rejoining enzyme (polynucleotide ligase) of mammalian cells requires ATP as a cofactor (Lindahl and Edelman, 1968). Double-strand breaks are generally regarded to be lethal to microorganisms (Freifelder, 1966), but there are some data suggesting that rejoining of double-strand scissions may be possible (Kitayama and Matsuyama, 1968). Both degradation of DNA (Emmerson and Howard-Flanders, 1965) and repair replication (Painter and Cleaver, 1967) have been observed in X-irradiated cells. Inasmuch as it is generally believed that the damage induced by ionizing radiation involves a DNA strand break, the incision step (see Section III,D) may not be required. Nevertheless, Dalrymple *et al.* (1969) have obtained evidence that nucleolytic enzymes markedly increase the number of DNA breaks which appear after ir-

radiation; these authors, therefore, believe that ionizing radiation produces not only single- and double-strand breaks in DNA, but also bond distortions which are liable to attack by nucleolytic enzymes. Since this enzymatic production of breaks seems to be energy dependent (Dalrymple *et al.*, 1969), the presence of an ATP-dependent endonuclease is suggested. In bacterial cells such an enzyme has been found (Anai *et al.*, 1970a,b). Provided there is a connection between DNA breaks and chromosome breaks, these findings may provide an explanation for the fact that production of chromosomal aberrations in plants by methylated oxypurines, such as 8-ethoxycaffeine, is dependent on oxidative phosphorylation (Kihlman, 1961, 1966).

2. *Alkylating Agents*

Breakage of DNA strands is also produced by alkylating agents, which like UV light and ionizing radiation are known as efficient chromosome breakers as well. It is the alkylation of heterocyclic nitrogen in DNA which is believed to be responsible for the genetic effects of these agents, and the most reactive site appears to be N-7 of guanine (Lawley and Brookes, 1963). After treatment with a difunctional alkylating agent, a large proportion of the alkylated guanine in DNA is cross-linked. This cross-linking may involve either adjacent guanines on the same strand or guanines on each of the complementary strands. Recent data suggest that between one-third and one-quarter of the cross-linkages are interstrand (Lawley *et al.*, 1969). The alkylated purines are unstable and tend to split off from the sugar-phosphate backbone; later fission of the sugar-phosphate chain at such apurinic sites follows at a slower rate. The process requires about 2000 hours to be completed (e.g., Lawley and Brookes, 1968). However, *in vitro* treatments with methyl methane sulfonate (MMS) result in a comparatively rapid appearance of single-strand DNA breaks (Strauss *et al.*, 1968).

Single-strand breaks also occur in alkylated DNA as a result of an enzymatic process. Thus, endonucleases have been discovered which produce single-strand breaks in alkylated DNA (Strauss and Robbins, 1968; Friedberg and Goldthwait, 1968). Nonconservative replication of DNA occurs in bacterial and mammalian cells after treatment with alkylating agents (Hanawalt and Haynes, 1965; Reiter *et al.*, 1967; Roberts *et al.*, 1968). Lawley and Brookes (1968) found that 400 nucleotides are removed for each cross-link induced in *E. coli* DNA by a difunctional mustard and about 10 for each alkylation by a monofunctional mustard. The same authors have found that, of the mustard gas-induced alkylations in bacterial DNA, all the diguanyl derivatives but only a fraction of

the monoalkylations can be enzymatically removed (Lawley and Brookes, 1965). Data obtained in experiments with microorganisms suggest that the system involved in the repair of the DNA lesions produced by a monofunctional alkylating agent such as MMS is partly different from that involved in the repair of the damage produced by a difunctional agent such as nitrogen mustard (HN2). On the other hand, there is evidence that damage caused by MMS is repaired by the same mechanism as X-ray-induced damage, whereas the mechanism by which HN2-induced damage is repaired is similar to that involved in the repair of UV-damage to DNA (Brendel *et al.*, 1970). These findings "are consistent with physicochemical data indicating that both MMS and X-rays produce single strand breaks . . . and that both mustard gas analogues and UV produce intrastrand cross-links in DNA . . ." (Brendel *et al.*, 1970).

3. *Accumulation of Single-Strand Breaks in DNA as a Result of Thymine Starvation*

An accumulation of single-strand DNA breaks has been observed in a thymine-requiring strain of *E. coli* after thymine starvation (Freifelder, 1969). Previously, a similar accumulation of single-strand DNA breaks was found after induction of a thymine-less state in nonmutant cells of *E. coli* by 5-fluorodeoxyuridine (FUdR), an inhibitor of thymidylic acid synthesis (Mennigmann and Szybalski, 1962).

In Freifelder's experiments, breaks did not arise in connection with DNA replication, and consequently they could not be the result of an incomplete synthesis of DNA. Freifelder suggested that the breaks are produced by an unknown physiological process and that thymine starvation results in the production of a substance which inhibits the polynucleotide ligase reaction required for the repair of the breaks. He further suggested that the inhibitor is deoxyadenosine triphosphate (dATP), which is known to accumulate in *E. coli* as a result of thymine starvation (Munch-Petersen and Neuhard, 1964); dATP acts as an inhibitor of purified *E. coli* ligase when assayed *in vitro* (I. R. Lehman, quoted in Freifelder, 1969).

4. *Exchanges between DNA Duplexes*

During the last decade it has become increasingly clear that breakage and rejoining of DNA molecules is involved in genetic recombination in both microorganisms and macroorganisms (for detailed discussion and references, see Taylor, 1967; Whitehouse, 1970). The data further

suggest that the recombination process is closely associated with DNA repair. At least the incision (endonuclease) and rejoining (ligase) steps seem to be involved; whether the excision and base replacement steps are also required is less certain.

Evidence for exchanges between radiation-damaged DNA molecules has recently been provided by Howard-Flanders and his collaborators in experiments with an excision-deficient strain of *E. coli* (Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968). Because of the stability of UV-induced dimers in this strain, these workers were able to study the replication of bacterial DNA containing a known number of dimers. It was found that replication of a DNA molecule containing dimers results in gaps in the newly synthesized strands at positions opposite to the dimers. It was further found that genetic exchanges occurred between sister duplexes when they contained dimers and opposing gaps. The authors suggested that recombination is promoted by the free ends at the gaps in the newly synthesized strands. As a result of the sister exchanges, the newly synthesized fragments can be assembled into a functional genome. Thus, the process represents a genetic recovery mechanism. It has been termed "recombinational repair" to distinguish it from excision repair, which removes pyrmidine dimers before replication. Since the type of excision repair processes operating on UV-induced pyrimidine dimers apparently is very similar to that operating on the diguanyl products produced by difunctional alkylating agents (see Section IV,A,2), it would be interesting to know whether the process of recombinational repair occurs when the parental DNA contains diguanyl products instead of pyrimidine dimers.

5. *Properties of DNA Molecules Significant for Patterns of Chromosome Breakage and Rejoining*

It is appropriate to conclude this subsection by summarizing some properties of DNA molecules which are of significance for cytological results to be described later. It should be noted that the two polynucleotide chains in the DNA double helix have opposite polarity as they run in opposite directions relative to the 3′- and 5′-deoxyribose–phosphate linkages (Fig. 6). As a result, the joining of broken polynucleotide chains is restricted, i.e., joining is possible only between ends where the chains run in the same direction. Another significant feature of DNA molecules is that the two polynucleotide chains in the double helix are held together by hydrogen bonds between complementary pairs of bases (Fig. 6). A consequence of this is that although breaks may occur in both strands, the molecule as a whole does not break. It has been calculated

that DNA molecules which suffer from simultaneous breaks in both strands may maintain their integrity even when the distance between the breaks is less than twenty complementary base pairs (Tomizawa and Ogawa, 1968); on the other hand, the molecule is broken when two single-strand breaks arise at a distance of five nucleotides or less (van der Schans, 1969). Provided that the broken sister strands remain hydrogen bonded to each other, breaks stand a good chance of eventually being repaired.

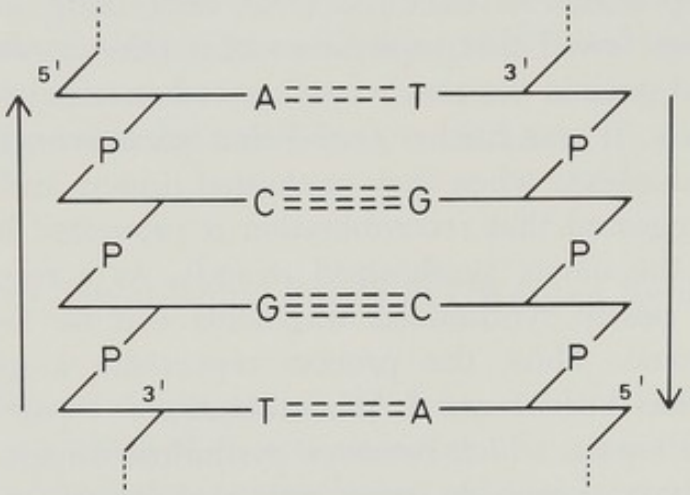


FIG. 6. Diagrammatic representation of part of a DNA molecule, showing hydrogen bonds between complementary bases and opposite polarity of strands. A = adenine, C = cytosine, G = guanine, and T = thymine. P stands for phosphate, and 3′ and 5′ refer to carbon atoms in the deoxyribose moieties.

B. Ultrastructure and Molecular Mechanisms Involved in Chromosome Breakage and Rejoining

1. *Unit of Breakage as Evidenced by Exchange Formation in Prophase*

It was mentioned in Section II,A that exchanges which appear to involve chromosome units smaller than a chromatid are obtained when cells in prophase are irradiated with ionizing radiation or treated with certain chemicals. Although this fact has been regarded as supporting a multineme model of chromosome structure, it is equally compatible with a folded-fiber model (DuPraw, 1970).

A way to discover the true nature of subchromatid exchanges is to find out what type of aberration they give rise to in the second (T2) division after treatment. If the exchanges are between true half-chroma-

tids, they should appear as exchanges of the chromatid type in the T2 division; on the other hand, if they are masked chromatid exchanges, they should give rise to chromosome-type exchanges in the T2 division.

This question was first investigated by Östergren and Wakonig (1954), who studied the nature of subchromatid exchanges induced by coumarin in root tips of *Allium cepa.* Simultaneously with and subsequent to the coumarin treatment, the roots were exposed to colchicine. As a result, cells which divided during the combined coumarin–colchicine treatment were tetraploid during the T2 division and could easily be distinguished from those remaining in interphase during the treatment. The majority of the aberrations observed by Östergren and Wakonig during the first division after treatment (T1 division) were subchromatid exchanges and attached fragments. The aberrations observed in tetraploid cells during the second division after treatment were almost exclusively of the chromosome type, which indicated that the exchanges in the T1 division were in fact between whole chromatids.

Later, Peacock (1961) and Heddle (quoted in Brinkley and Humphrey, 1969) claimed that they had found chromatid-type aberrations when cells containing subchromatid aberrations in the T1 division divided for the second time. LaCour and Rutishauser (1954) attributed the failure of Östergren and Wakonig to find chromatid exchanges in T2 cells to difficulties in the timing of the experiments. Nevertheless, this criticism should not apply to the experiments of Kihlman and Hartley (1967a) and Kihlman (1970a).

The experimental material in the latter studies were root tips of *Vicia faba* and *Allium cepa;* cells were labeled by making them tetraploid with colchicine or binucleate with theophylline. The latter method was used mainly because Nuti Ronchi (1968) had claimed that the colchicine treatment was responsible for the occurrence of chromosome-type aberrations in the T2 division. The results of the experiments of Kihlman and Hartley (1967a) and Kihlman (1970a) were in complete agreement with those of Östergren and Wakonig, i.e., the aberrations in the tetraploid or binucleate T2 cells were all of the chromosome type.

In the experiments of Kihlman (1970a) special attention was paid to the morphology of the subchromatid exchanges during the T1 division; if the structures involved in the exchange were not exact half-chromatids, there would be no reason to expect the exchanges to be chromatid-type aberrations in the T2 division. It was found that the T1 aberrations have very different morphologies, possibly depending on how late in prophase they are induced. Thus, there appears to be a gradual transition from a configuration where the two exchanging chromatids are connected only by two thin threads (Fig. 7a) to a configura-

tion where the material involved in the exchange comprises the greater part of the diameter of each chromatid (Fig. 7c). This suggests that a variable amount of material is shared by the two chromatids involved in a subchromatid exchange. Although it is not possible to see the fine structure of this material with the light microscope, it seems very likely that the material is composed of a varying number of submicroscopic strands, and that these strands are identical with the 250 Å strands seen in electron micrographs of chromosomes prepared by the surface-

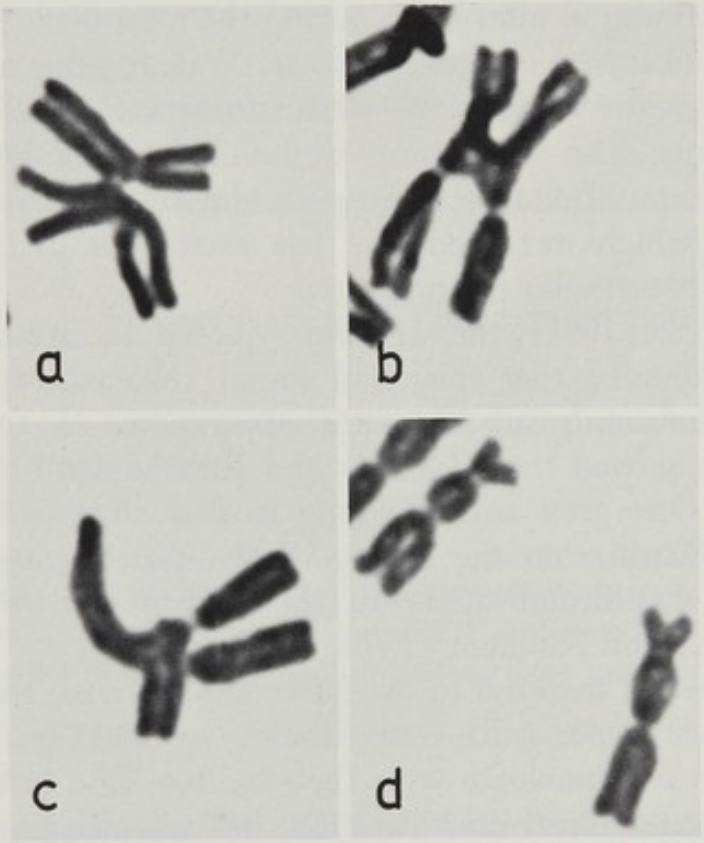


FIG. 7. Metaphase appearances of subchromatid interchanges (a,b,c) and intrachanges (d) in *Allium* root tips. (From Kihlman, 1970a.)

spreading and critical point drying technique (see Section III,B). On *a priori* grounds these strands might represent the units of a multineme chromatid, but such multiple lengths of fiber connecting chromatids at a point of exchange can also represent parts of a single, long folded fiber (DuPraw, 1965b, 1966, 1968). The fact that the subchromatid exchanges of the T1 division appear as chromosome-type exchanges in the T2 division supports the folded fiber model.

Figure 8 demonstrates how the configurations observed in T1 (subchromatid exchanges) can lead to the aberrations seen in T2 (chromosome-type exchanges) in chromosomes having a folded-fiber structure.

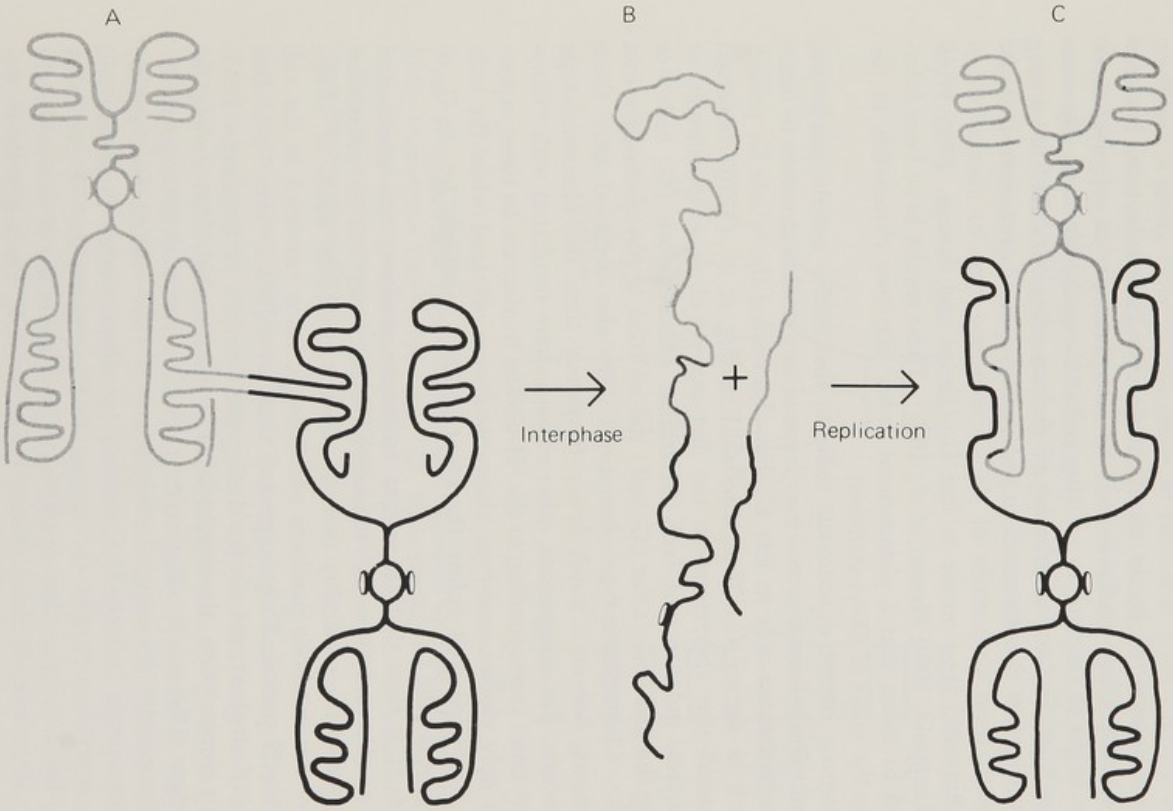


FIG. 8. Configuration of subchromatid exchanges at T1 and T2 division according to the folded-fiber model. When induced at prophase, exchanges between 250 Å fibers give rise to side-arm bridges at T1 anaphase, and to dicentrics at the T2 division. [From DuPraw (1970). *DNA and Chromosomes.* Holt, Rinehart & Winston, Inc.]

2. *Reactions of Chromosomes to Ionizing Radiation in the G_1 Stage of Interphase*

There appears to be an approximate coincidence in time between the onset of DNA synthesis and the interphase period when chromosomes begin to respond to ionizing radiation as double units. By combining X-irradiation and labeling of chromosomes with ^{3}H-TdR during S, investigators demonstrated in autoradiographic experiments that the transition from aberrations of the chromosome type to aberrations of the chromatid type occurs in late G_1 (Evans and Savage, 1963; Wolff and Luippold, 1964; Monesi *et al.*, 1967). These results have been interpreted to mean that a chromosome splits into two chromatids in G_1, before the onset of DNA synthesis.

However, recent experiments indicate that under certain conditions, a nonduplicated chromosome may respond as a double structure to ionizing radiation not only in late G_1, but also during the whole G_1 period, as well as during G_0. Thus, Lindahl-Kiessling *et al.* (1970) observed chromatid-type aberrations in the first mitosis of *in vitro* cultures of leukocytes, pulse-treated with ^{3}H-uridine either before (G_0 cells) or during the first hours after (G_1 cell) the addition of phytohemagglutinin (PHA). Chromosome-type aberrations were also obtained after these treatments, but at a much lower frequency than the chromatid-type aberrations. For comparison, other cultures were irradiated with ^{60}Co-γ-rays at times corresponding to the ^{3}H-uridine treatments. In these experiments only chromosome-type aberrations were obtained when the cells were irradiated well before the onset of DNA synthesis, i.e., in G_0 or early and middle G_1.

Why is it then that ^{3}H-uridine treatment produces this unexpected effect in G_0 and G_1 cells? Lindahl-Kiessling *et al.* (1970) considered and rejected the possibility that the effect is caused by traces of ^{3}H-uridine or its metabolic products which might have remained in the cell until the S period. They suggested that β-irradiation from ^{3}H-uridine produced single-strand breaks during the synthesis of messenger RNA. During transcription, the segments of single-stranded DNA coding for messenger RNA are in close contact with the tritiated RNA precursor, thereby greatly increasing the likelihood of DNA strand breakage by the low energy β-irradiation emitted by tritium. RNA synthesis, and hence irradiation, would be concentrated in certain loci of the DNA molecules, a situation that would favor the production of several breaks within these loci. Because, during RNA synthesis, the segments of single-stranded template DNA are probably separated from, and *not* hydrogen bonded to, the complementary segments of the sister DNA strand,

it seems likely that a piece of single-stranded DNA between two nearby breaks may detach and get lost (Fig. 9). The gap thus formed would constitute a much more severe lesion in the DNA than a single strand break in one sugar-phosphate chain. If the damage persisted until S, chromatid-type aberrations would be formed by misreplication of DNA molecules containing single-strand gaps.

In γ-irradiated cells, the probability of several breaks being concentrated in the same locus is small, and even smaller is the probability

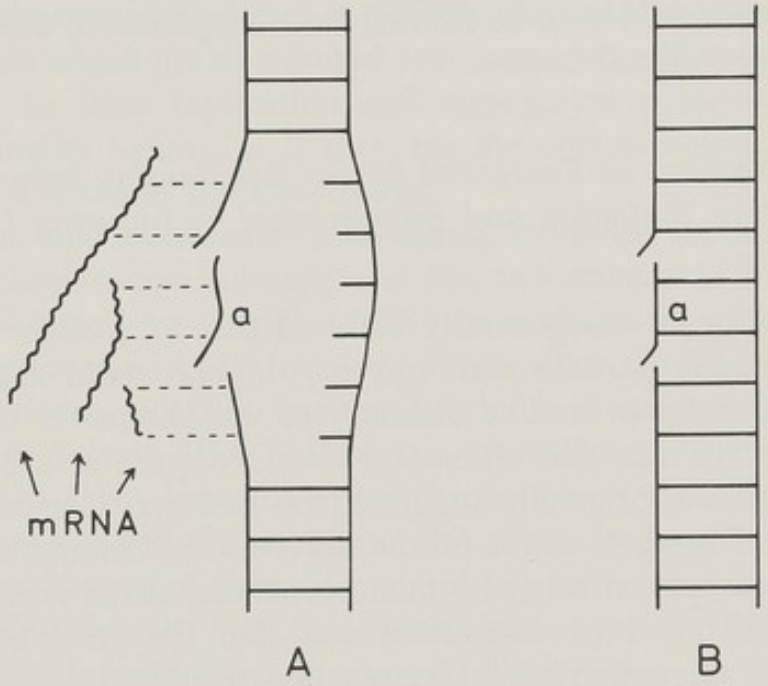


FIG. 9. Single-strand breaks induced in DNA either by β-rays from ^{3}H-uridine during transcription (A) or by γ-rays (B). Since the sister strands are separated in A, the piece of single-stranded DNA (*a*) between the two adjacent breaks is likely to be lost, whereas in (B) the broken ends are kept in place by hydrogen bonds between sister strands.

that such adjacent breaks will occur in a segment where the sister strands are not held together by hydrogen bonds. If breaks occur in a region of DNA molecule not involved in RNA synthesis, the hydrogen bonds between the sister strands will serve to keep the broken ends and DNA segments in position for repair (compare Section IV,A,5). Irradiation of G_1 cells with γ-rays would therefore be expected to produce exclusively chromosome-type aberrations if the only DNA strand breaks that are not immediately and successfully repaired are those which occur simultaneously at the same or adjacent loci in both strands of the DNA molecule.

Possibly significant for this concept is the finding of Dewey and Miller

(1969) that G_1 chromosomes in Chinese hamster cells pretreated with colcemid can respond as double structures to X-irradiation. In the absence of colcemid, 90% of the exchanges induced in mitotic and G_1 cells were of the chromosome type. The frequency of chromosome-type exchanges was not affected by the colcemid pretreatment, but the total frequency of exchanges was doubled due to the appearance of chromatid-type exchanges. These findings become understandable if the colcemid pretreatment causes the free ends of radiation-induced single-strand DNA breaks to separate, thereby delaying repair until S. Chromatid aberrations would then be formed by misreplication, as suggested for the ^{3}H-uridine-induced lesions.

3. *Unit of Breakage as Evidenced by the Relationship between the LET of Ionization Radiation and Effectiveness in Inducing Chromosomal Aberrations*

Until recently, it was generally believed that to produce a chromatid break, an ionizing particle must traverse the chromatid and produce at least 17 ionizations in it. The diameter of the chromatid was supposed to be 0.1 μ. The idea that chromatid breaks require a large number of energy-loss events, originally suggested by Lea and Catcheside (1942), was supported by their works on the induction of chromatid aberrations in *Tradescantia* pollen tubes by monochromatic soft X-rays (Catcheside and Lea, 1943); a key observation was that the yield of aberrations increases with increasing LET (linear energy transfer).

More recently, the effects of soft X-rays (1.5 keV or 8.3 Å and 3 keV or 4.1 Å) on pollen tube chromosomes have been reexamined by Neary *et al.* (1964). They found that chromatid aberrations are, in fact, quite efficiently induced by 1.5 keV X-rays, which activate photoelectrons with path lengths of only about 0.05 μ. The authors concluded that "there is no longer any basis for the estimate that some 17 'ionizations' are required for efficient 'breakage' of a chromatid." They believe that most of the known facts can be accounted for by a model of aberration formation in which a primary lesion in a chromosome is produced by one energy-loss event in a structure with a thickness of the order of 10 Å; the aberration itself would be the result of an exchange between two such lesions. Although their experiments did not reveal the chemical nature of the structure in which the primary lesion is produced, the authors concluded that it must be quite fundamental and were tempted to identify it with a DNA double helix forming the skeletal axis of a chromatid. Subsequently, this model of aberration formation has been further supported by new data regarding the dependence of aberration

yield on dose, LET, and modifying agents (Neary, 1965; Neary and Savage, 1966; Neary *et al.*, 1967).

The *in vivo* results of Neary and co-workers also provide an important confirmation that a chromatid axis has the dimensions described for type A and type B chromatin fibers by DuPraw and Bahr (1969; see also DuPraw, 1970).

4. *Restricted Rejoining of Chromosome Subunits*

The experiments of Taylor *et al.* (1957) showed that there are chromosome subunits which are distributed semiconservatively between daughter chromatids. In their replication and segregation patterns, these chromosome subunits behave as if they are the complementary polynucleotide chains of a single DNA molecule.

Additional, and even more convincing, evidence for an identity between the chromosome subunits and the two strands of a DNA double helix was obtained by Taylor (1958) in a study on patterns of sister chromatid exchange. At the second mitosis after labeling with ^{3}H-TdR, each chromosome generally has one labeled and one unlabeled chromatid (compare Section III,C). However, even though a semiconservative segregation of DNA between sister chromatids is the rule, switches of label from one sister chromatid to the other frequently occur. These reciprocal exchanges of sister chromatid segments were used by Taylor (1958) to demonstrate that the chromosome subunits, like the strands of a DNA double helix, have directional polarity.

Taylor's experiments were performed with root tips of *Bellevalia romana* and colchicine was used to make the T2 division tetraploid. In the tetraploid T2 metaphases, Taylor found that sister chromatid exchanges frequently occurred at the same locus in two of the four homologous chromosomes; these were called twin exchanges, in contrast to the more usual single exchanges involving only one of the four homologous chromosomes. (No evidence was found for the occurrence of half-chromatid exchanges.) Taylor argued that the relative frequencies of single and twin exchanges would depend on whether rejoining between subunits is restricted or occurs at random. If the chromosome subunits are the complementary strands of a DNA double helix, with opposite chemical polarity, a symmetrical exchange could occur only between an old and a new subunit; this is because after replication a new (labeled) subunit in one sister chromatid must have the same polarity as an old (unlabeled) subunit in the other (compare Section IV,A,5). If rejoining is restricted in this way, every exchange occurring during the first replication cycle must appear as a twin exchange in the tetra-

ploid T2 metaphases; by contrast, exchanges occurring in the second cycle must appear as singles. Because there are twice as many chromosomes present during the second replication cycle, the ratio between singles and twins should be 2:1. On the other hand, if no polarity exists and rejoining between subunits occurs at random, there should be ten singles to one twin (Taylor, 1958). The ratios observed by Taylor varied between 1:2 and 2:1. Consequently, it is clear from these data that the frequency of twin exchanges is much higher than that expected on the basis of random rejoining; at the same time, the ratios fit the expectation for restricted rejoining quite well. These results provide strong support for the idea that the chromosomal subunits are identical with the complementary strands of a single DNA molecule per chromatid.

Not all subsequent data have been in agreement with Taylor's results. However, in a recent analysis of sister chromatid exchanges in the metacentric chromosomes of *Vicia faba*, Geard and Peacock (1969) observed frequencies of single and twin exchanges which were consistent with a ratio of 2:1. Possible sources of errors were considered and found to be without significant influence on the results. The authors concluded that their data ". . . support the postulate that the two replication subunits of the chromosome are dissimilar." However, they added cautiously that ". . . although the restrictions to reunion of subunits are precisely those which are imposed by the structure of the DNA molecule, these experiments have not demonstrated that the exchanges between sister chromatids do in fact involve the DNA moiety of the chromosome" (Geard and Peacock, 1969).

Another, independent way to test the proposition that segregating chromosomal subunits are dissimilar has been worked out by Brewen and Peacock (1969). This test involves experimental analysis of segregation of ^{3}H-TdR-labeled chromosomal subunits in dicentric chromosomes of Chinese hamster cell cultures. The dicentrics, which were studied in the second (T2) division after labeling, were obtained after suppression of anaphase with colcemid and arose by replication of X-ray-induced isolocus breaks with proximal sister union.

Production of "mirror-image" dicentrics is illustrated in Fig. 10, which also shows the two possible types of end-to-end association of the segregating chromosomal subunits. If rejoining is restricted so that labeled subunits always associate with labeled, and unlabeled with unlabeled, then a dicentric will be produced in which all the label in the region between the centromeres will be in one chromatid (Fig. 10A). On the other hand, if a labeled subunit always associates with an unlabeled one, the resulting dicentric will have an exchange of label at the mid-

point between the centromeres (Fig. 10B). Equal frequencies of these two types of dicentrics should result if the subunits rejoin randomly.

The great majority of "mirror-image" dicentrics analyzed by Brewen and Peacock had all the label conserved in one sister chromatid in the intercentromeric region. The authors concluded that ". . . the end-to-end association of the subunits is not random but is strictly preferential. The data suggest that the functional subunit in the formation of these particular chromosomal aberrations might be single polynucleotide chains of deoxyribonucleic acid, although other molecular species cannot be disregarded" (Brewen and Peacock, 1969).

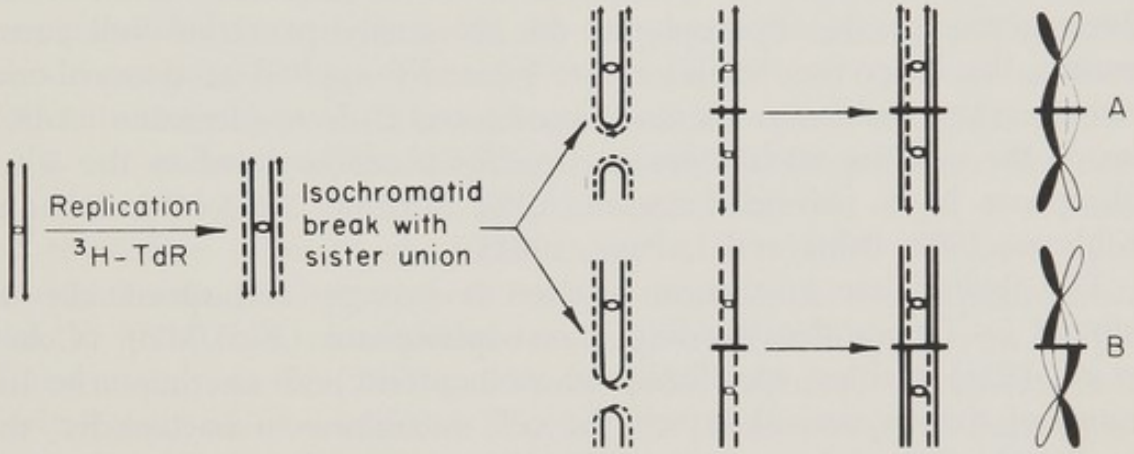


FIG. 10. Diagrammatic representation showing the two alternate types (A and B) of end-to-end association of chromosomal subunits in an isochromatid break and the resulting segregation pattern of subunits in dicentrics of the T2 division. The dotted line and the solid chromosome regions indicate the presence of ^{3}H-TdR and label, respectively. (From Brewen and Peacock, 1969.)

5. *Molecular Species Involved in Chromosome Breakage as Evidenced by the Effects of Inhibitors of Deoxyribonucleotide Synthesis on Chromosome Structure*

A method of testing the proposition that DNA is the molecular species responsible for the structural integrity of chromosomes is by exposing cells to inhibitors of DNA synthesis. To ensure specificity, only substances should be used which inhibit reactions subsequent to the separation of the RNA and DNA synthetic pathways. Appropriate inhibitors would be those which affect (1) the ribonucleotide reductase reactions, by which deoxyribonucleotides are formed from ribonucleotides, (2) the thymidylate synthetase reaction, by which deoxyuridylic acid is methylated to thymidylic acid, (3) the kinase reactions, by which deoxyribonucleoside triphosphates (i.e., the immediate precursors of DNA) are formed from the corresponding deoxyribonucleoside mono- and di-

phosphates, (4) the various functions of DNA polymerase (polymerization, exonuclease activity), and (5) the polynucleotide ligase reaction, by which two segments of an interrupted strand in a DNA duplex are covalently joined (esterification of an internally located 3′-hydroxyl group with an adjacent 5′-phosphomonoester).

Substances known to inhibit the ribonucleotide reductase reactions are hydroxyurea (HU) (Young and Hodas, 1964; Young *et al.*, 1967; Adams *et al.*, 1966; Adams and Lindsay, 1967; Turner *et al.*, 1966; Krakoff *et al.*, 1968; Elford, 1968), arabinosyl adenine 5′-triphosphate or arabinosyl ATP (York and LePage, 1966; Moore and Cohen, 1967) and 2′-deoxyadenosine 5′-triphosphate or dATP (Reichard *et al.*, 1961). Because nucleoside triphosphates do not easily penetrate cell membranes, the latter two inhibitors are generally applied as deoxyribonucleosides, i.e., arabinosyl adenine (ara-A) and 2′-deoxyadenosine (AdR); inside the cell the nucleosides are readily phosphorylated to the 5′-triphosphate level (Munch-Petersen, 1960; Klenow, 1962; Odmark and Kihlman, 1965; Brink and LePage, 1964).

The thymidylate synthetase reaction is strongly and specifically inhibited by 5-fluorodeoxyuridine 5′-monophosphate (F-dUMP) (Cohen *et al.*, 1958) and less specifically by aminopterin and amethopterin. Because of the impermeability of the cell membrane to nucleotides, the nucleoside 5-fluorodeoxyuridine (FUdR) is generally used rather than F-dUMP.

Selective inhibitors of the kinase reactions are scarce, but recently two such inhibitors of the thymidylate kinase reaction have been described. These are 5′-deoxy-5′-fluorothymidine (Langen and Kowollik, 1968) and 5′-deoxythymidine (Kára and Duschinsky, 1969).

The ATP-requiring polynucleotide ligase in *E. coli* infected with bacteriophage T4 is inhibited by dATP (Weiss *et al.*, 1968), and so is apparently the NAD-requiring ligase in uninfected *E. coli* (I. R. Lehman, quoted in Freifelder, 1969). DeoxyATP does not inhibit the ATP-requiring ligase in mammalian cells (Lindahl and Edelman, 1968).

Of the substances mentioned above, inhibitors of the ribonucleotide reductase and the thymidylate synthetase reactions have been studied in cytological experiments. Taylor *et al.* (1962) reported that treatment of *Vicia faba* roots with low concentrations (around $10^{-6}M$) of FUdR resulted in chromatid aberrations of a very characteristic type. Whereas the aberrations most commonly found in plant roots after exposure to radiation or radiomimetic chemicals are chromatid inter- and intrachanges, FUdR produced almost exclusively gaps and open chromatid breaks. The aberrations began to appear in meta- and anaphase about 3 hours after the beginning of the treatment, which indicated that they

were produced at the end of interphase. Furthermore, the chromosome-breaking effect of FUdR could be prevented by exposing the roots to thymidine, simultaneously with or subsequent to the FUdR treatment (Taylor *et al.*, 1962; Kihlman, 1962). In order to be effective, the concentration of thymidine had to be about 100 times higher than that of FUdR.

Taylor *et al.* (1962) also obtained striking results when FUdR treatments were combined with X-irradiation. They found that the frequencies of gaps and open breaks obtained after a given dose of X-rays were drastically increased when the roots were exposed to FUdR before and during the X-irradiation. The increase in gaps and open breaks was paralleled by a decrease in the frequency of exchanges. Thymidine also counteracted this effect of FUdR, which was observed at lower concentrations than the chromosome-breaking effect of FUdR alone.

In cell cultures of the Chinese hamster, FUdR produces the same type of cytological effect as in plant cells, and the effect is reversed by thymidine (Hsu *et al.*, 1964; Ockey *et al.*, 1968). However, there is an interesting difference between the effects in the two types of material. In plant cells, chromosome breakage by FUdR appears to be produced mainly at G_2 (Bell and Wolff, 1964), although chromosomes in early S may also be affected (Taylor, 1963). However, in experiments with Chinese hamster fibroblasts, aberrations were produced only in cells which were treated during the DNA synthesis period (Ockey *et al.*, 1968). Early S cells appeared to be particularly sensitive.

Other inhibitors of deoxyribonucleotide synthesis have subsequently been found to produce the same characteristic cytological effect as FUdR. In root tips of *Vicia faba*, Taylor obtained this effect with aminopterin (Taylor, 1963). It has further been observed both in plant and mammalian cells after treatments with AdR (Kihlman, 1963; Kihlman *et al.*, 1963), ara-A (Nichols, 1964; Rao and Natarajan, 1965; Kihlman and Odmark, 1966), and hydroxyurea (e.g., Kihlman *et al.*, 1966; Yu and Sinclair, 1968) (see Fig. 11). For hydroxyurea (HU), the same differences were observed between the effects in Chinese hamster cells and in *Vicia* root tips as were previously described for FUdR (Kihlman and Sturelid, unpublished). Aminopterin, AdR, ara-A, and HU had effects similar to that of FUdR not only when used alone, but also when combined with X-rays (Taylor, 1963; Kihlman and Hartley, 1968). The effect in combination with X-rays was analyzed in more detail by Kihlman and Hartley (1968) using HU as an inhibitor. The increase in the frequencies of gaps and breaks proved to be most striking when HU was given during the last 2.5 hours before fixation (metaphase). An equally drastic increase in the frequencies of gaps and breaks was

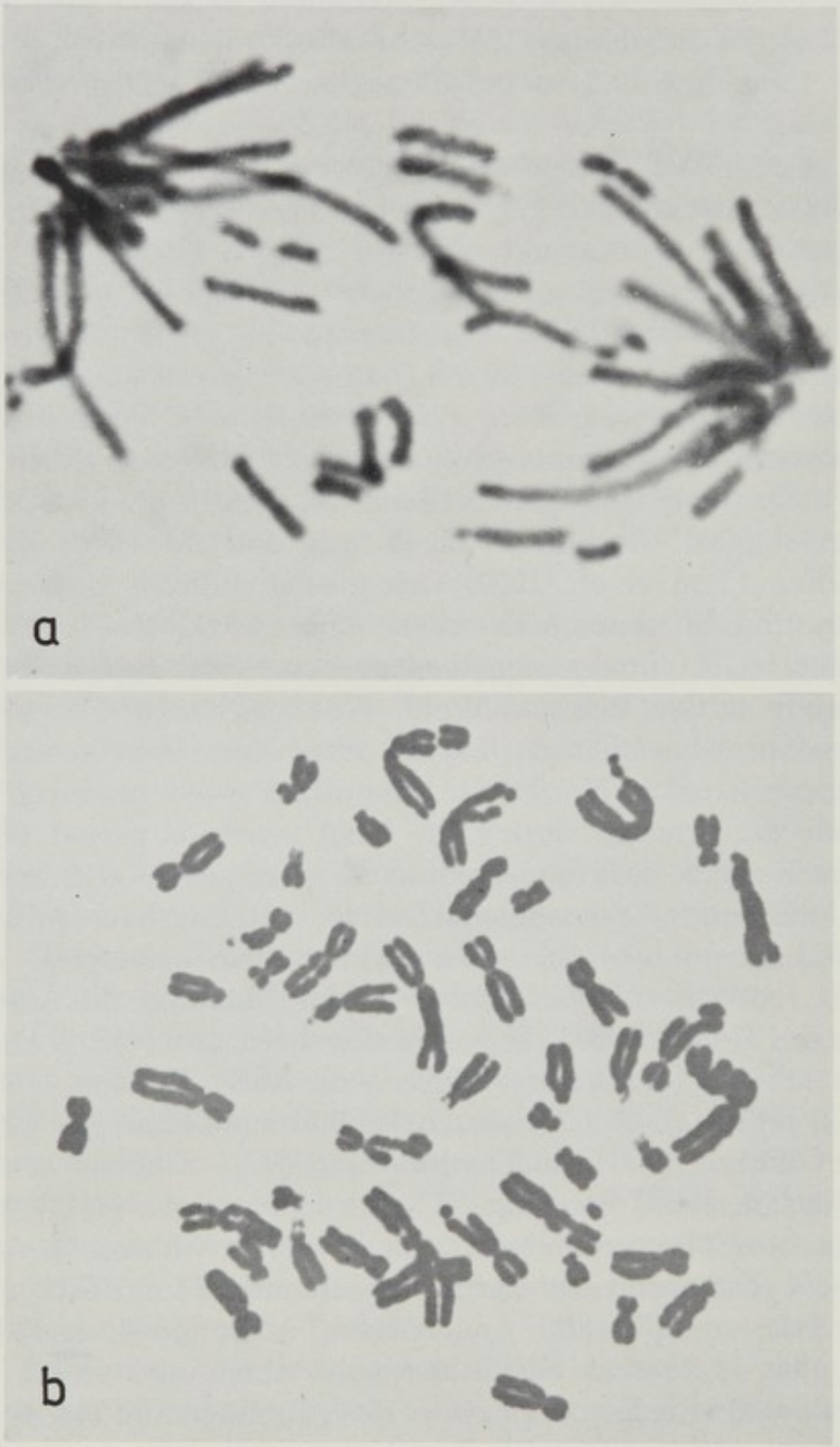


FIG. 11. (a) Hydroxyurea-induced fragmentation of anaphase chromosomes in *Vicia faba*. (From Kihlman *et al.*, 1966.) (b) 2′-Deoxyadenosine-induced breaks and gaps in a human tissue culture cell, arrested at metaphase by colchicine. (From Kihlman *et al.*, 1963.)

obtained when the roots were first treated with certain radiomimetic chemicals (nitrogen mustard, maleic hydroxide, streptonigrin, and phleomycin) and then exposed to HU during the last 2.5 hours before fixation.

Ito *et al.* (1967) studied DNA synthesis during meiotic prophase in *in vitro* cultures of lily anther cells. These authors found that when AdR was added to meiotic cells in mid-zygonema, the prophase chromosomes were drastically fragmented. Inhibition of DNA synthesis by AdR during late zygonema or early pachynema did not affect the first meiotic division, but it resulted in fragmentation of chromosomes during the second division.

The effects of inhibitors of deoxyribonucleotide synthesis on chromosome structure have been interpreted somewhat differently by different authors. Taylor *et al.* (1962) suggested that the chromosome-breaking effect of FUdR results from an FUdR-induced inhibition of DNA synthesis in cells which are just finishing S phase when they are affected by the analog. However, the experiments of Bell and Wolff (1964) demonstrated that FUdR produces chromosome damage in bean roots mainly during the G_2 stage, and so do AdR (Kihlman and Hartley, 1967b) and HU (Kihlman and Sturelid, unpublished). Therefore, if DNA replication is the target for production of chromosomal aberrations in *Vicia* by these inhibitors, it must be quantitatively different from the DNA synthesis in S, inasmuch as it cannot be detected by conventional autoradiographic methods (Kihlman, 1966). By contrast, the data obtained in experiments with Chinese hamster cells are in good agreement with Taylor's hypothesis.

The hypothesis of Taylor *et al.* (1962) was that treatment with FUdR results in chromosome damage because this treatment disturbs the normal replication of DNA. The same authors suggested that FUdR increases the frequency of gaps and open breaks after X-irradiation because the inhibitor prevents the repair of X-ray-induced damage in chromosomal DNA. The experimental results of Kihlman and Hartley (1968) were similarly interpreted. These authors concluded that X-rays and radiomimetic chemicals produce lesions in chromosomes, the repair of which requires deoxyribonucleotides; when there is a deficiency of deoxyribonucleotides as a result of treatment with an inhibitor, repair tends to be incomplete (Kihlman and Hartley, 1968).

Although most authors agree that chromosome damage produced by inhibitors of deoxyribonucleotide synthesis alone results from the DNA precursor deficiency caused by treatment with these agents, hypotheses differ regarding the mechanism by which the deficiency results in chromosomal aberrations. According to Ahnström and Natarajan (1966),

chromosome breakage by FUdR is the result of a reversal of the DNA polymerase reaction caused by the deficiency of deoxyribonucleoside triphosphates. In the hypotheses of other investigators two main ideas may be distinguished. One is that the inhibitors produce chromosome damage because they prevent repair of breaks or lesions which occur as normal events at certain stages of the mitotic cycle (Kihlman and Hartley, 1967b). It has been suggested that DNA strand breaks occur in connection with transcription (Pauling and Hanawalt, 1965) or normal replication (Hanawalt, 1968). The other concept is that chromosome damage occurs because the inhibitors suppress the replication of a special type of DNA (satellite DNA?). This DNA would normally be synthesized at a stage of the cell cycle subsequent to S; although it must be necessary for the structural integrity of chromosomes, this DNA represents only a small fraction of total chromosomal DNA (Kihlman, 1966; Kihlman and Hartley, 1968).

These hypotheses were proposed as two alternative explanations for the chromosome damage produced by inhibitors alone during the G_2 period of interphase. Chromosome fragmentation induced by AdR in meitotic prophase is believed by Ito *et al.* (1967) to be the result of an inhibited synthesis of axial DNA, whereas chromosome breakage in anaphase II results from a disturbance of normal repair mechanisms associated with genetic crossing over.

6. Alkylation of DNA and Chromosomal Aberrations

The early studies of Ford (1949) and Revell (1953) have shown that nitrogen mustard and di(2,3-epoxypropyl)ether produce few or no aberrations in bean roots during the first 8 hours after the beginning of treatment. The maximum frequency of aberrations was obtained in roots fixed 24 to 48 hours after treatment, and all aberrations were of the chromatid type. Subchromatid and chromosome-type aberrations were not found. Furthermore, aberrations were localized to heterochromatic segments of the chromosome. These findings have since been confirmed by other authors and were shown to be valid for alkylating agents in general.

A detailed analysis of the effects of nitrogen mustard (HN2) on chromosome structure in root tips of *Vicia faba* has recently been published by Evans and Scott (1969). In their autoradiographic experiments, they determined the interphase sensitivity to the chromosome-breaking effect of HN2 by using ^{3}H-TdR to label chromosomes in S cells. At the first mitosis following treatment, Evans and Scott found chromatid aberrations in cells which were in G_1 or S when exposed to HN2. By contrast, cells exposed to HN2 when in G_2 appeared normal at the first mitosis,

but developed chromatid-type aberrations at the second mitosis after treatment; in fact, the frequency of aberrations observed at the T2 mitosis was considerably higher than that observed at the first. These results led the authors to conclude that "although HN2 produces lesions in chromosomes independently of cell phase at the time of treatment, structural changes are only produced when the cells undergo DNA synthesis." Evans and Scott (1969) also suggested that chromatid aberrations arise "as a result of misreplication of DNA at the sites of alkylation" and concluded that "the high frequency of intrachange relative to interchange aberrations and the localization of aberrations to the heterochromatic regions of the chromosomes are consequences of the requirement for simultaneous replication in chromosome regions that are involved in aberration." Although alkylation of DNA can produce spontaneous DNA chain breakage without the intervention of enzymatic processes (compare Section IV,A,2), it is a slow process, requiring 2000 hours or more (Lawley and Brookes, 1968); therefore, it can hardly be responsible for the formation of aberrations which are observed 10–72 hours after treatment (Evans and Scott, 1969). For this reason, aberration formation as a result of treatment with alkylating agents probably involves enzymatic breakage and rejoining of DNA.

According to Lawley and Brookes (1963), an important lesion induced by difunctional alkylating agents in DNA is cross-linkage of guanine moieties on opposite strands of the DNA double helix. However, Evans and Scott (1969) showed that of the various types of aberration produced by HN2, only the isochromatid aberrations could have arisen by misreplication of DNA at the site of such an interstrand cross-link. On the other hand, all the observed aberration types could have arisen by misreplication at two closely associated alkylated sites, each of which would involve only one strand of a DNA duplex. Supposedly misreplication would be followed by excision and replacement of the damaged bases on the "old" strand at these sites; this process would then yield only chromatid-type aberrations, in agreement with the observed facts (Fig. 12). To account for the fact that difunctional agents are considerably more effective in producing aberrations than are monofunctional ones (Loveless, 1951; see also Sturelid, quoted in Kihlman, 1970b), Evans and Scott suggested that cross-linking of DNA to other chromosome constituents may be of importance.

A somewhat different interpretation is suggested by the similarities between the DNA damage caused by UV light and by difunctional alkylating agents. Recent studies by Lawley *et al.* (1969) on the effects of difunctional sulfur mustard on DNA indicates that three out of four cross-linkages are intrastrand. Apparently, enzymes involved in repair

and replication react very much in the same way to the diguanyl derivative present in intrastrand cross-linkage as to the pyrimidine dimers produced by UV light (compare Section IV,A,2). Rupp and Howard-

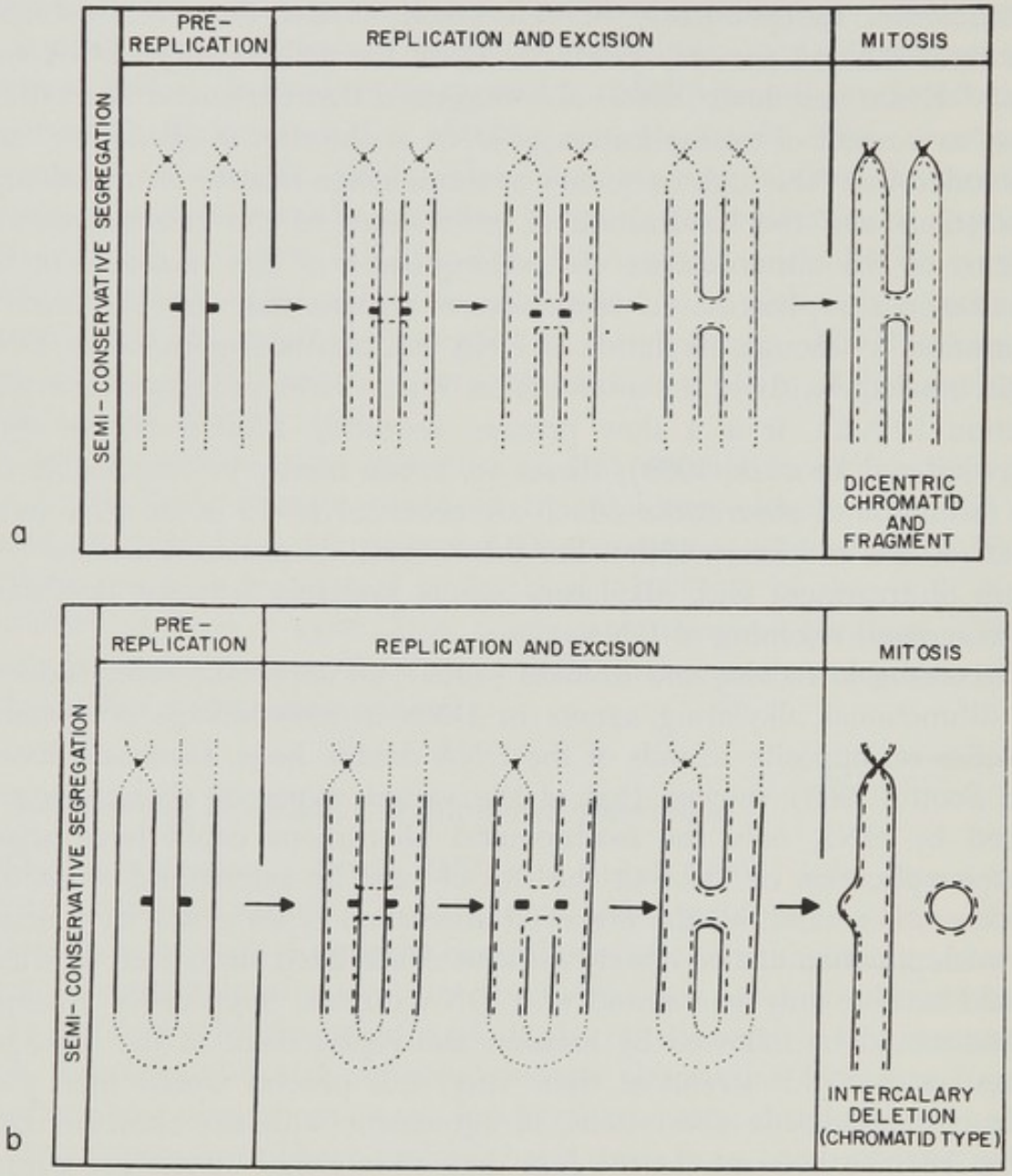


FIG. 12. Diagram illustrating the production of chromatid interchange (a) and intrachange (b) by misreplication at the region of two monofunctional alkylations. (From Evans and Scott, 1969.)

Flanders (1968) have shown that if dimers are not removed before the stage of DNA synthesis (S), gaps will be formed in the newly synthesized strands at positions opposite the dimers. Exchanges between DNA duplexes are promoted by these gaps. Postreplicative excision of the dimers would cause double-strand breaks. That this type of mecha-

nism may be involved in the production of chromosomal aberrations by UV is indicated by the fact that cells in S at the time of irradiation "suffer about ten times the chromosomal damage of cells in G_1 and G_2 phases" and by the fact that few if any chromosome-type aberrations are induced by UV light (Humphrey *et al.*, 1963). A similar mechanism operating on diguanyl products in DNA may be responsible for the production of chromatid-type aberrations after treatments with difunctional alkylating agents.

In summary, it appears that agents which produce chromosomal aberrations belong to two main categories: (1) those which induce spontaneous or enzymatic DNA strand breakage and/or interfere with DNA synthesis by modifying the DNA duplex chemically or physically; and (2) those which inhibit the repair of DNA strand breaks or the joining of newly synthesized DNA segments by suppressing the formation of one or several of the deoxyribonucleoside triphosphates required for DNA replication, or by inhibiting the polynucleotide ligase reaction.

7. *Repair Replication and Chromosomal Aberrations*

The observations described in Sections III and IV indicate that the 250 Å chromosomal fibers are the units of chromosome breakage and rejoining, and that DNA is the molecular species primarily involved in these processes. Beyond this, the data suggest that exchange-type aberrations are formed by misreplication and/or misrepair of DNA at two closely associated sites of damage.

The idea that repair processes similar to those responsible for dark repair of DNA are involved in aberration formation was probably first expressed by Evans (1966) to account for the effects of fractionated X-ray exposures. Evans believes that the primary lesions produced in the chromosomes by X-radiation are not open breaks. These lesions supposedly undergo a repair process which may be identical with the dark repair process known from studies with microorganisms. Chromosome aberrations theoretically could arise by "misrepair" (cross polymerization) when two lesions occur close enough together. According to the hypothesis, no aberrations would be formed in the absence of repair; Feulgen-negative zones which are usually called "gaps" would represent unrepaired lesions (see Fig. 1a,b).

An important consequence of the misrepair hypothesis is that "aberrations are something that the cell makes and not something that the radiation makes" (Evans, 1968). Another important consequence is that "chromosome exchanges, whether induced by physical or chemical agents or occurring spontaneously at mitosis, giving mitotic crossing over, or at

meiosis, giving meiotic crossing over, are all basically similar events ultimately utilizing the same pathways in the cell" (Evans, 1966). Similar ideas have been expressed by Kihlman and Hartley (1967b, 1968), and by Dubinin (1969; Dubinin and Soyfer, 1969).

Not all facts fit this concept of the molecular mechanism of aberration formation quite as well as the facts so far presented. For instance, inhibitors such as hydroxyurea and FUdR, which are supposed to produce or enhance chromosome damage by preventing repair, do not inhibit repair replication or unscheduled DNA synthesis (Painter and Cleaver, 1967; Djordjevic and Tolmach, 1967; Evans and Norman, 1968; Cleaver, 1968a, 1969a). According to Cleaver (1968a) the reason why these inhibitors do not affect repair replication is probably that the number of deoxyribonucleotides required for repair is so small that they can be produced even in the presence of the inhibitors. It should also be borne in mind that the X-ray dosages used in studies on repair replication and unscheduled DNA synthesis are about 1000 times higher than those used in studies on chromosome breakage. At such high dosages, there is a possibility that the deoxyribonucleotide supply is increased by DNA degradation. Furthermore, detection of repair replication and unscheduled DNA synthesis only means that nonconservative DNA replication has taken place, but it does not provide any evidence for a complete or successful repair. In fact, the recent results of Rasmussen *et al.* (1970) have shown that at high levels of damage the repair system is not capable of producing functional DNA. Recently, Djordjevic *et al.* (1969) found that hydroxyurea does inhibit spontaneous unscheduled DNA synthesis during the G_1 phase in HeLa cells. According to these authors, it is conceivable that this spontaneous unscheduled DNA synthesis accounts for the repair of lesions which have been induced as a part of the normal metabolic activity of the cells.

The idea that processes like those responsible for dark repair of DNA are involved in aberration formation has been criticized by Wolff and Scott (1969). Among the reasons why they regard this proposal as unlikely are the following: (1) Rejoining of chromosome breaks is affected by inhibitors of protein synthesis but not by inhibitors of DNA synthesis; (2) X-irradiation of the two cell lines DON and B14FAF of the Chinese hamster produces the same relative frequency of exchange-type aberrations, although the two cell lines differ strongly in their ability to perform unscheduled DNA synthesis; (3) leukocytes from a patient with Xeroderma pigmentosum responded to X-irradiation with a similar frequency of exchange-type aberrations as leukocytes from normal persons, although Xeroderma cells do not exhibit unscheduled DNA synthesis; (4) exchange-type aberrations are produced by X-rays in root tip cells of *Vicia*

faba, although unscheduled DNA synthesis has not been demonstrated in these cells; and (5) caffeine, which inhibits dark repair in bacteria, does not affect the rejoining of chromosome breaks in *Vicia*.

In the studies of Wolff and his collaborators on the effects of caffeine and inhibitors of protein and DNA synthesis on rejoining, root meristem cells of *Vicia faba* were irradiated in the G_1 phase (soaked seeds) with fractionated X-ray dosages. The time interval between the dosages was 75–110 minutes; under normal conditions, this time is long enough to prevent the primary lesions produced by the first dose from interacting with those produced by the second, and the yield of exchange-type aberrations is the sum of the exchanges induced by the two dosages. Inhibition of the process which make the primary lesions unable to take part in exchange formation makes it possible for the lesions produced by the first dose to interact with those produced by the second; in this case, the yield of exchange aberrations becomes proportional to the square of the total dose administered.

Wolff (1960) found that administering protein inhibitors, such as chloramphenicol and aureomycin, between the dosages makes the yield of exchange aberrations proportional to the square of the total dose. By contrast, an aberration yield equal to the sum of the aberrations produced by the two dosages was obtained when the seeds were treated between the dosages with FUdR (Bell and Wolff, 1964) or caffeine (Wolff and Scott, 1969). The authors assumed that the primary lesion is a break and interpreted their experimental results to mean that protein synthesis, but not DNA synthesis, is required for the rejoining or repair of chromosome breaks.

As a possible explanation for the finding that protein inhibitors prolong the time during which lesions produced by different dose fractions can interact to form exchange aberrations, Wolff (1960) suggested that these inhibitors suppress the formation of an enzyme involved in the rejoining process. Because normal semiconservative DNA synthesis did not occur at the time of the treatments, the author concluded that the enzyme could not be required for making DNA. The possibility that the enzyme might be required for nonconservative DNA repair replication was not considered simply because this process was unknown at that time. It is a fact, however, that inhibitors of protein synthesis also are strong inhibitors of DNA synthesis (e.g., Zampetti-Bosseler *et al.*, 1969; Wolff, 1969). It has also been found that chloramphenicol (one of the inhibitors used by Wolff) does not affect nuclear protein synthesis (Kuehl, 1969).

The negative results with FUdR (Bell and Wolff, 1964) are also inconclusive. Treatment with FUdR may inhibit the formation of thymidylic

acid, but in order to become an inhibitor, FUdR must be phosphorylated to 5-fluorodeoxyuridine 5′-phosphate; this phosphorylation is catalyzed by the enzyme thymidine kinase (Okazaki and Kornberg, 1964). Although there does not seem to be any information about the activity of this enzyme in soaked bean seeds, it has been demonstrated by several authors (e.g., Adams, 1969; Klevecz, 1969) that the activity of thymidine kinase is very low during the G_1 stage of interphase. Consequently, it is possible that FUdR was found inactive because it was not phosphorylated. The drastic increase in the frequency of gaps and breaks obtained when X-irradiated cells were treated with FUdR and other inhibitors of deoxyribonucleotide synthesis during the last 2–3 hours before metaphase (Taylor *et al.*, 1962; Kihlman and Hartley, 1968) suggests that these inhibitors suppress the repair of radiation damage. Evidence for an inhibitory effect of FUdR on the repair of γ-induced lesions has also been obtained by Yamamoto and Yamaguchi (1969a), who studied the effect in barley root-tip cells exposed to FUdR during the G_2 stage of interphase.

Finally, the evidence obtained in the caffeine experiments is also somewhat doubtful. In mammalian cells, caffeine is not an inhibitor of repair replication or unscheduled DNA synthesis (Cleaver, 1969a); rather, it is believed that "caffeine interferes specifically with a step associated with semiconservative DNA replication in irradiated cells" (Cleaver and Thomas, 1969). However, it is true that the experiments of Wolff and Scott were not performed with mammalian cells but with bean root cells in the G_1 stage of interphase, and it is also true that root-tip cells and mammalian cells in tissue cultures respond very differently to the chromosome-breaking effect of caffeine (Kihlman, 1952, 1970b; Ostertag, 1966). Evidence for an inhibitory effect of caffeine on the repair of γ-ray-induced chromosomal damage in barley has recently been obtained by Yamamoto and Yamaguchi (1969b) and by Ahnström and Natarajan (1971). In the experiments of Yamamoto and Yamaguchi, root-tip cells were irradiated and treated with caffeine when in the G_2 stage of interphase. In the experiments of Ahnström and Natarajan, resting seeds of 13% moisture (cells in G_0) were first irradiated and then soaked for 5 hours in 0.1% caffeine. In both studies, the frequency of chromosomal aberrations was drastically increased by the caffeine treatment.

Wolff and Scott (1969) found that exchange-type aberrations were produced by X-rays in cells which had little or no capacity to perform repair replication or unscheduled DNA synthesis when irradiated. Unfortunately, one of the systems used by the authors has since proved to be a rather unhappy choice. As first demonstrated by Cleaver (1968b),

skin fibroblasts of patients with the disease Xeroderma pigmentosum do show reduced amounts of DNA repair replication *in vitro* after UV irradiation. However, Cleaver (1969b) has subsequently shown that such fibroblasts can perform normal amounts of unscheduled DNA synthesis after irradiation with X-rays. Because the chromosome-type exchanges obtained by Wolff and Scott in lymphocytes from a Xeroderma pigmentosum patient were induced by X-rays, normal repair would be expected.

Two observations remain: (1) X-rays produce the same relative frequencies of exchanges in two strains of Chinese hamster cells which differ markedly in their ability to perform unscheduled DNA synthesis; and (2) unscheduled DNA synthesis cannot be induced by irradiating root-tips cells of *Vicia faba*, a classic material for the study of exchange-type aberrations. These observations, together with the fact that repair replication is not suppressed by inhibitors of deoxyribonucleotide synthesis, even though treatment with these agents results in a decreased survival of X-irradiated mammalian cells (Weiss and Tolmach, 1967; Sinclair, 1968), and in a drastic increase in the frequencies of radiation-induced and chemically induced gaps and breaks, suggest that the processes which influence survival and which are involved in the repair of chromosome damage are different from those responsible for unscheduled DNA synthesis and repair replication observed after heavy dosages of X-rays (cf. Section III,D). Whether this difference is a qualitative one and not just quantitative remains to be seen.

V. Concluding Remarks

At present it is a reasonable and useful working hypothesis that DNA is the key substance in chromosome breakage and rejoining, and that essentially the same biochemical mechanisms are involved in dark repair of DNA, in genetic recombination, and in the formation of chromosomal aberrations. This hypothesis has been the main theme of my article. My decision to concentrate on a working hypothesis, rather than simply reviewing current ideas about the mechanisms of chromosome breakage and rejoining, was influenced by my belief that such an approach is more likely to stimulate discussion and research within this rather neglected field of molecular genetics.

REFERENCES

Abuelo, J. G., and Moore, D. E. (1969). *J. Cell Biol.* **41**: 73.
Adams. R. L. P. (1969). *Exp. Cell Res.* **56**: 49.
Adams, R. L. P., and Lindsay, J. G. (1967). *J. Biol. Chem.* **242**: 1314.

Adams, R. L. P., Abrams, R., and Lieberman, I. (1966). *J. Biol. Chem.* **241**: 903.
Ahnström, G., and Natarajan, A. T. (1966). *Hereditas* **54**: 379.
Ahnström, G., and Natarajan, A. T. (1971). *Int. J. Radiat. Biol.* (in press).
Alfert, M. (1955). *Fine Struct. Cells, Symp., Leiden, 1954*, p. 157.
Anai, M., Hirahashi, T., and Takagi, Y. (1970a). *J. Biol. Chem.* **245**: 767.
Anai, M., Hirahashi, T., Yamanaka, M., and Takagi, Y. (1970b). *J. Biol. Chem.* **245**: 775.
Anderson, T. F. (1950). *Trans. N.Y. Acad. Sci.* **13**: 130.
Aoki, S., Boyce, R. P., and Howard-Flanders, P. (1966). *Nature (London)* **209**: 686.
Bell, S., and Wolff, S. (1964). *Proc. Nat. Acad. Sci. U.S.* **51**: 195.
Bloch, D. P., Macquigg, R. A., Brack, S. D., and Wu, J.-R. (1967). *J. Cell Biol.* **33**: 451.
Boyce, R. P. (1966). *Nature (London)* **209**: 688.
Brendel, M., Khan, N. A., and Haynes, R. H. (1970). *Mol. Gen. Genet.* **106**: 289.
Brewen, J. G., and Brock, R. D. (1968). *Mutat. Res.* **6**: 245.
Brewen, J. G., and Peacock, W. J. (1969). *Proc. Nat. Acad. Sci. U.S.* **62**: 389.
Brink, J. J., and LePage, G. A. (1964). *Cancer Res.* **24**: 312.
Brinkley, B. R., and Humphrey, R. M. (1969). *J. Cell Biol.* **42**: 827.
Callan, H. G., and MacGregor, H. C. (1958). *Nature (London)* **181**: 1479.
Catcheside, D. G., and Lea, D. E. (1943). *J. Genet.* **45**: 186.
Chayen, J., LaCour, L. F., and Gahan, P. S. (1957). *Nature (London)* **180**: 652.
Cleaver, J. E. (1968a). *Biophys. J.* **8**: 775.
Cleaver, J. E. (1968b). *Nature (London)* **218**: 652.
Cleaver, J. E. (1969a). *Radiat. Res.* **37**: 334.
Cleaver, J. E. (1969b). *Proc. Nat. Acad. Sci. U.S.* **63**: 428.
Cleaver, J. E., and Painter, R. B. (1968). *Biochim. Biophys. Acta* **161**: 552.
Cleaver, J. E., and Thomas, G. H. (1969). *Biochem. Biophys. Res. Commun.* **36**: 203.
Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J. (1958). *Proc. Nat. Acad. Sci. U.S.* **44**: 1004.
Coleman, J. R., and Okada, S. (1968). *Biophys. J.* **8**: 1098.
Comings, D. E., and Okada, T. A. (1969). *J. Cell Biol.* **43**: 25a.
Corry, P. M., and Cole, A. (1968). *Radiat. Res.* **36**: 528.
Crouse, H. V. (1954). *Science* **119**: 485.
Dalrymple, G. V., Sanders, J. L., Moss, A. J., Jr., Baker, M. L., and Wilkinson, K. P. (1969). *Biochem. Biophys. Res. Commun.* **36**: 284.
Das, N. K., and Alfert, M. (1968). *Exp. Cell Res.* **49**: 51.
Dean, C. J., Ormerod, M. G., Serianni, R. W., and Alexander, P. (1969). *Nature (London)* **222**: 1042.
Dewey, W. C., and Miller, H. H. (1969). *Exp. Cell Res.* **57**: 63.
Djordjevic, B., and Tolmach, L. J. (1967). *Radiat. Res.* **32**: 327.
Djordjevic, B., Evans, R. G., Perez, A. G., and Weill, M. K. (1969). *Nature (London)* **224**: 803.
Dubinin, N. P. (1969). *Proc. 12th Inter. Congr. Genet., Tokyo*, 1968 **3**: 79.
Dubinin, N. P., and Soyfer, V. N. (1969). *Mutat. Res.* **8**: 353.
DuPraw, E. J. (1965a). *Proc. Nat. Acad. Sci. U.S.* **53**: 161.
DuPraw, E. J. (1965b). *Nature (London)* **206**: 338.

DuPraw, E. J. (1966). *Nature (London)* **209**: 577.
DuPraw, E. J. (1968). "Cell and Molecular Biology," 739 pp. Academic Press, New York.
DuPraw, E. J. (1969). Preprint.
DuPraw, E. J. (1970). "DNA and Chromosomes," 340 pp. Holt, Rinehart & Winston, New York.
DuPraw, E. J., and Bahr, G. F. (1969). *Acta Cytol.* **13**: 188.
Elford, H. L. (1968). *Biochem. Biophys. Res. Commun.* 33: 129.
Emmerson, P. T., and Howard-Flanders, P. (1965). *Biochem. Biophys. Res. Commun.* **18**: 24.
Evans, H. J. (1962). *Int. Rev. Cytol.* **13**: 221.
Evans, H. J. (1964). *Exp. Cell Res.* **35**: 381.
Evans, H. J. (1966). *In* "Genetical Aspects of Radiosensitivity: Mechanisms of Repair," p. 31. Int. At. Energy Agency, Vienna.
Evans, H. J. (1968). *Brookhaven Symp. Biol.* **20**: 111.
Evans, H. J., and Neary, G. J. (1958). *Radiat. Res.* **9**: 111.
Evans, H. J., and Savage, J. R. K. (1963). *J. Cell Biol.* **18**: 525.
Evans, H. J., and Scott, D. (1969). *Proc. Roy. Soc., Ser. B* **173**: 491.
Evans, R. G., and Norman, A. (1968). *Nature (London)* **217**: 455.
Ford, C. E. (1949). *Proc. 8th Int. Congr. Genet., Stockholm, 1948* p. 570.
Frederic, J. (1969). *Chromosoma* **28**: 199.
Freifelder, D. (1966). *Radiat. Res. Suppl.* **6**: 80.
Freifelder, D. (1969). *J. Mol. Biol.* **45**: 1.
Friedberg, E. C., and Goldthwait, D. A. (1968). *Cold Spring Harbor Symp. Quant. Biol.* **33**: 271.
Gahan, P. S. (1965). *Exp. Cell Res.* **39**: 136.
Gall, J. G. (1963a). *Nature (London)* **198**: 36.
Gall, J. G. (1963b). *Science* **139**: 120.
Gall, J. G. (1966). *Chromosoma* **20**: 221.
Geard, C. R., and Peacock, W. J. (1969). *Mutat. Res.* **7**: 215.
Grumbach, M. M., Morishima, A., and Taylor J. H. (1963). *Proc. Nat. Acad. Sci. U.S.* **49**: 581.
Gurley, L. R., and Hardin, J. M. (1968). *Arch. Biochem. Biophys.* **128**: 285.
Habener, J. E., Bynum, B. S., and Shack, J. (1969). *Biochim. Biophys. Acta* **195**: 484.
Hahn, G. M., Yang, S.-J., and Parker, V. (1968). *Nature (London)* **220**: 1142.
Hanawalt, P. C. (1968). *In* "Photophysiology" (A. C. Giese, ed.), Vol. 4, p. 203. Academic Press, New York.
Hanawalt, P. C., and Haynes, R. H. (1965). *Biochem. Biophys. Res. Commun.* **19**: 462.
Haynes, R. H. (1966). *Radiat. Res. Suppl.* **6**: 1.
Heddle, J. A., and Bodycote, D. J. (1970). *Mutat. Res.* **9**: 117.
Hill, M. (1967). *Int. J. Radiat. Biol.* **13**: 199.
Hill, M. (1969). *Int. J. Radiat. Biol.* **15**: 483.
Howard, A., and Pelc, S. R. (1953). Symp. Chromosome Breakage. *Heredity* **6** (Suppl.): 261.
Howard-Flanders, P., Rupp, W. D., Wilkins, B. M., and Cole, R. S. (1968). *Cold Spring Harbor Symp. Quant. Biol.* **33**: 195.
Hsu, T. C., Humphrey, R. M., and Somers, C. E. (1964). *J. Nat. Cancer Inst.* **32**: 839.

Hsu, W. S. (1968). *Chromosoma* **24**: 288.
Huberman, J. A., and Attardi, G. (1966). *J. Cell Biol.* **31**: 95.
Huberman, J. A., and Riggs, A. D. (1966). *Proc. Nat. Acad. Sci. U.S.* **55**: 599.
Huberman, J. A., and Riggs, A. D. (1968). *J. Mol. Biol.* **32**: 327.
Humphrey, R. M., Dewey, W. C., and Cork, A. (1963). *Radiat. Res.* **19**: 247.
Humphrey, R. M., Steward, D. L., and Sedita, B. A. (1968). *Mutat. Res.* **6**: 459.
Hutchinson, F., and Koehrlin, W. (1967). *Radiat. Res.* **31**: 547.
Ito, M., Hotta, Y., and Stern, H. (1967). *Develop. Biol.* **16**: 54.
Jockusch, B. M., Brown, D. F., and Rusch, H. P. (1970). *Biochem. Biophys. Res. Commun.* **38**: 279.
Kanazir, D. T. (1969). *Progr. Nucl. Acid Res. Mol. Biol.* **9**: 117.
Kára, J., and Duschinsky, R. (1969). *Biochim. Biophys. Acta* **186**: 223.
Kelly, R. B., Atkinson, M. R., Huberman, J. A., and Kornberg, A. (1969). *Nature (London)* **224**: 495.
Kidwell, W. R., and Mueller, G. C. (1969). *Biochem. Biophys. Res. Commun.* **36**: 756.
Kihlman, B. A. (1952). *Symb. Bot. Upsal.* **11**(4): 1.
Kihlman, B. A. (1961). *Advan. Genet.* **10**: 1.
Kihlman, B. A. (1962). *Caryologia* **15**: 261.
Kihlman, B. A. (1963). *J. Cell. Comp. Physiol.* **62**: 267.
Kihlman, B. A. (1966). "Actions of Chemicals on Dividing Cells," 260 pp. Prentice-Hall, Englewood Cliffs, New Jersey.
Kihlman, B. A. (1970a). *Hereditas* **65**: 171.
Kihlman, B. A. (1970b). *In* "Chemical Mutagens: Principles and Methods for their Detection" (A. Hollaender, ed.). Plenum Press, New York.
Kihlman, B. A., and Hartley, B. (1967a). *Hereditas* **57**: 289.
Kihlman, B. A., and Hartley, B. (1967b). *Mutat. Res.* **4**: 771.
Kihlman, B. A., and Hartley, B. (1968). *Hereditas,* **59**: 439.
Kihlman, B. A., and Odmark, G. (1965). *Mutat. Res.* **2**: 494.
Kihlman, B. A., and Odmark, G. (1966). *Hereditas* **56**: 71.
Kihlman, B. A., Nichols, W. W., and Levan, A. (1963). *Hereditas* **50**: 139.
Kihlman, B. A., Eriksson, T., and Odmark, G. (1966). *Hereditas* **55**: 386.
Kihlman, B. A., Odmark, G., and Hartley, B. (1967). *Mutat. Res.* **4**: 783.
Kitayama, S., and Matsuyama, A. (1968). *Biochem. Biophys. Res. Commun.* **33**: 418.
Kleinschmidt, A. K., Lang, D., Jacherts, D., and Zahn, R. K. (1962). *Biochim. Biophys. Acta* **61**: 857.
Klenow, H. (1962). *Biochim. Biophys. Acta* **61**: 885.
Klevecz, R. R. (1969). *Science* **166**: 1536.
Krakoff, I. H., Brown, N. C., and Reichard, P. (1968). *Cancer Res.* **28**: 1559.
Kuehl, L. (1969). *J. Cell Biol.* **41**: 660.
LaCour, L. F., and Rutishauser, A. (1954). *Chromosoma* **6**: 696.
LaCour, L. F., Chayen, J., and Gahan, P. S. (1958). *Exp. Cell Res.* **14**: 469.
Lajtha, L. G. (1963). *J. Cell Comp. Physiol.* **62** (Suppl. 1): 143.
Langen, P., and Kowollik, G. (1968). *Eur. J. Biochem.* **6**: 344.
Lawley, P. D., and Brookes, P. (1963). *Exp. Cell Res. Suppl.* **9**: 512.
Lawley, P. D., and Brookes, P. (1965). *Nature (London)* **206**: 480.
Lawley, P. D., and Brookes, P. (1968). *Biochem. J.* **109**: 433.
Lawley, P. D., Lethbridge, J. H., Edwards, P. A., and Shooter, K. V. (1969). *J. Mol. Biol.* **39**: 181.

Lea, D. E., and Catcheside, D. G. (1942). *J. Genet.* **44**: 216.
Lett, J. T., Caldwell, I., Dean, C. J., and Alexander, P. (1967). *Nature (London)* **214**: 790.
Levan, A., and Tjio, J. H. (1948). *Hereditas* **34**: 453.
Lima-de-Faria, A. (1959). *J. Biophys. Biochem. Cytol.* **6**: 457.
Lindahl, T., and Edelman, G. M. (1968). *Proc. Nat. Acad. Sci. U.S.* **61**: 680.
Lindahl-Kiessling, K., Santesson, B., and Böök, J. A. (1970). *Chromosoma* **31**: 280.
Loveless, A. (1951). *Nature (London)* **167**: 338.
MacGregor, H. C., and Callan, H. G. (1962). *Quart. J. Microsc. Sci.* **103**: 173.
McLeish, J. (1959). *Chromosoma* **10**: 686.
Maio, J. J., and Schildkraut, C. L. (1967). *J. Mol. Biol.* **24**: 29.
Matsudaira, H., Nakagawa, C.. and Bannai, S. (1969). *Int. J. Radiat. Biol.* **15**: 575.
Matsudaira, H., Furuno, I., and Otsuka, H. (1970). *Int. J. Radiat. Biol.* **17**: 339.
Mattingly, E. (1967). *Mutat. Res.* **4**: 51.
Mennigmann, H. D., and Szybalski, W. (1962). *Biochem. Biophys. Res. Commun.* **9**: 398.
Miller, O. L. (1965). *Nat. Cancer Inst. Monogr.* **18**: 79.
Monesi, V., Crippa, M., and Zito-Bignami, R. (1967). *Chromosoma* **21**: 369.
Moore, E. C., and Cohen, S. S. (1967). *J. Biol. Chem.* **242**: 2116.
Mueller, G. C. (1969). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**: 1780.
Munch-Petersen, A. (1960). *Biochem. Biophys. Res. Commun.* **3**: 392.
Munch-Petersen, A., and Neuhard, J. (1964). *Biochim. Biophys. Acta* **80**: 542.
Neary, G. J. (1965). *Int. J. Radiat. Biol.* **9**: 477.
Neary, G. J., and Savage, J. R. K. (1966). *Int. J. Radiat. Biol.* **11**: 209.
Neary, G. J., Savage, J. R. K., and Evans, H. J. (1964). *Int. J. Radiat. Biol.* **8**: 1.
Neary, G. J., Preston, R. J., and Savage, J. R. K. (1967). *Int. J. Radiat. Biol.* **12**: 317.
Nichols, W. W. (1964). *Cancer Res.* **24**: 1502.
Nozawa, R., and Mizuno, D. (1969). *Proc. Nat. Acad. Sci. U.S.* **63**: 904.
Nuti Ronchi, V. (1968). Istituto di Genetica dell' Università di Pisa. Report for the academic year 1967–1968, p. 19.
Ockey, C. H., Hsu, T. C., and Richardson, L. C. (1968). *J. Nat. Cancer Inst.* **40**: 465.
Odmark, G., and Kihlman, B. A. (1965). *Mutat. Res.* **2**: 274.
Oishi, M. (1968a). *Proc. Nat. Acad. Sci. U.S.* **60**: 329.
Oishi, M. (1968b). *Proc. Nat. Acad. Sci. U.S.* **60**: 691.
Oishi, M. (1968c). *Proc. Nat. Acad. Sci. U.S.* **60**: 1000.
Okazaki, R., and Kornberg, A. (1964). *J. Biol. Chem.* **239**: 269.
Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., and Sugino, A. (1968). *Proc. Nat. Acad. Sci. U.S.* **59**: 598.
Olivera, B. M., and Lehman, I. R. (1967). *Proc. Nat. Acad. Sci. U.S.* **57**: 1700.
Östergren, G. (1948). *Bot. Notis.* p. 376.
Östergren, G., and Wakonig, T. (1954). *Bot. Notis.* p. 357.
Ostertag, W. (1966). *Mutat. Res.* **3**: 249.
Painter, R. B. (1968a). *J. Cell Biol.* **39**: 102a.
Painter, R. B. (1968b). *Radiat. Res.* **35**: 513.
Painter, R. B., and Cleaver, J. E. (1967). *Nature (London)* **216**: 369.
Painter, R. B., and Cleaver, J. E. (1969). *Radiat. Res.* **37**: 451.
Painter, R. B., and Schaefer, A. (1969). *Nature (London)* **221**: 1215.

Painter, R. B., Jermany, D. A., and Rasmussen, R. E. (1966). *J. Mol. Biol.* **17**: 47.
Pauling, C., and Hanawalt, P. (1965). *Proc. Nat. Acad. Sci. U.S.* **54**: 1728.
Peacock, W. J. (1961). *Nature (London)* **191**: 832.
Pettijohn, D. E., and Hanawalt, P. C. (1964). *J. Mol. Biol.* **9**: 395.
Rao, R. N., and Natarajan, A. T. (1965). *Cancer Res.* **25**: 1761.
Rasmussen, R. E., and Painter, R. B. (1964). *Nature (London)* **203**: 1360.
Rasmussen, R. E., and Painter, R. B. (1966). *J. Cell Biol.* **29**: 11.
Rasmussen, R. E., Reisner, B. L., and Painter, R. B. (1970). *Int. J. Radiat. Biol.* **17**: 285.
Reichard, P., Canellakis, Z. N., and Canellakis, E. S. (1961). *J. Biol. Chem.* **236**: 2514.
Reiter, H., Strauss, B., Robbins, M., and Marone, R. (1967). *J. Bacteriol.* **93**: 1056.
Revell, S. H. (1953). Symp. Chromosome Breakage. *Heredity* **6** (Suppl.): 107.
Revell, S. H. (1955). *In* "Proceedings of the Radiobiology Symposium, Liege, 1954" (Z. M. Bacq and P. Alexander, eds.), p. 243. Butterworth, London.
Revell, S. H. (1959). *Proc. Roy. Soc. Ser. B* **150**: 563.
Riggs, A. D., and Huberman, J. A. (1967). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **26**: 868.
Ris, H. (1967). *In* "Regulation of Nucleic Acid and Protein Biosynthesis," Proc. Int. Symp., Lunteren, Neth. (V. V. Konigsberger and L. Bosch, eds.), p. 11. Elsevier, Amsterdam.
Robbins, E., and Borun, T. W. (1967). *Proc. Nat. Acad. Sci. U.S.* **57**: 409.
Roberts, J. J., Crathorn, A. R., and Brent, T. P. (1968). *Nature (London)* **218**: 970.
Rupp, W. D., and Howard-Flanders, P. (1968). *J. Mol. Biol.* **31**: 291.
Salzman, N. P., Moore, D. E., and Mendelsohn, J. (1966). *Proc. Nat. Acad. Sci. U.S.* **56**: 1449.
Sasaki, M. S, and Norman, A. (1966). *Exp. Cell Res.* **44**: 642.
Savage, J. R. K. (1968). *In* "Neutron Irradiation of Seeds II," p. 9. Int. At. Energy Agency, Vienna.
Savage, J. R. K., Preston, R. J., and Neary, G. J. (1968). *Mutat. Res.* **5**: 47.
Sax, K. (1939). *Proc. Nat. Acad. Sci. U.S.* **25**: 225.
Sax, K. (1940). *Genetics* **25**: 41.
Sax, K. (1941). *Cold Spring Harbor Symp. Quant. Biol.* **9**: 93.
Sax, K., and King, E. D. (1955). *Proc. Nat. Acad. Sci. U.S.* **41**: 150.
Schandl, E. K., and Taylor, J. H. (1969). *Biochem. Biophys. Res. Commun.* **34**: 291.
Schwarzacher, H. G., and Schnedl, W. (1969). *Humangenetik* **8**: 75.
Scott, D., and Evans, H. J. (1967). *Mutat. Res.* **4**: 579.
Sinclair, W. K. (1968). *Cancer Res.* **28**: 198.
Smith, K. C. (1966). *Radiat. Res. Suppl.* **6**: 54.
Strauss, B., and Robbins, M. (1968). *Biochim. Biophys. Acta* **161**: 68.
Strauss, B., Coyle, M., and Robbins, M. (1968). *Cold Spring Harbor Symp. Quant. Biol.* **33**: 277.
Sugimoto, K., Okazaki, T., and Okazaki, R. (1968). *Proc. Nat. Acad. Sci. U.S.* **60**: 1356.
Taylor, J. H. (1958). *Genetics* **43**: 515.
Taylor, J. H. (1960). *J. Biophys. Biochem. Cytol.* **7**: 455.

Taylor, J. H. (1963). *J. Cell Comp. Physiol.* **62** (Suppl. 1): 73.
Taylor, J. H., ed. (1967). *In* "Molecular Genetics," Part II, p. 95. Academic Press, New York.
Taylor, J. H. (1968). *J. Mol. Biol.* **31**: 579.
Taylor, J. H. (1969). *Proc. 12th Int. Congr. Genet., Tokyo,* 1968 **3**: 177.
Taylor, J. H., and Straubing, N. (1968). *J. Cell Biol.* **39**: 134a.
Taylor, J. H., Woods, P. S., and Hughes, W. L. (1957). *Proc. Nat. Acad. Sci. U.S.* **43**: 122.
Taylor, J. H., Haut, W. F., and Tung, J. (1962). *Proc. Nat. Acad. Sci. U.S.* **48**: 190.
Tomizawa, J., and Ogawa, H. (1968). *Cold Spring Harbor Symp. Quant. Biol.* **33**: 243.
Turner, M. K., Abrams, R., and Lieberman, I. (1966). *J. Biol. Chem.* **241**: 5777.
van der Schans, G. P. (1969). *Int. J. Radiat. Biol.* **16**: 58.
Veatch, W., and Okada, S. (1969). *Biophys. J.* **9**: 330.
Weiss, B., Jacquemin-Sablon, A., Live, T. R., Fareed, G. C., and Richardson, C. C. (1968). *J. Biol. Chem.* **243**: 4543.
Weiss, B. G., and Tolmach, L. J. (1967). *Biophys. J.* **7**: 779.
Whitehouse, H. L. K. (1970). *Biol. Rev.* **45**: 265.
Wilson, G. B., Sparrow, A. H., and Pond, V. (1959). *Am. J. Botany* **46**: 309.
Wolfe, S. L. (1968). *J. Cell Biol.* **37**: 610.
Wolff, S. (1960). *Am. Naturalist* **94**: 85.
Wolff, S. (1963). "Radiation-induced Chromosome Aberrations." 304 pp. Columbia Univ. Press, New York.
Wolff, S. (1969). *Mutat. Res.* **8**: 207.
Wolff, S., and Luippold, H. E. (1964). *Exp. Cell Res.* **34**: 548.
Wolff, S., and Scott, D. (1969). *Exp. Cell Res.* **55**: 9.
Yamamoto, K., and Yamaguchi, H. (1969a). *Mutat. Res.* **8**: 424.
Yamamoto, K., and Yamaguchi, H. (1969b). *Mutat. Res.* **8**: 428.
York, J. L., and LePage, G. A. (1966). *Can. J. Biochem.* **44**: 19.
Young, C. W., and Hodas, S. (1964). *Science* **146**: 1172.
Young, C. W., Schochetman, G., and Karnofsky, D. A. (1967). *Cancer Res.* **27**: 526.
Yu, C. K., and Sinclair, W. K. (1968). *J. Cell. Physiol.* **72**: 39.
Yudelevich, A., Ginsberg, B., and Hurwitz, J. (1968). *Proc. Nat. Acad. Sci. U.S.* **61**: 1129.
Zampetti-Bosseler, F., Malpoix, P., and Fievez, M. (1969). *Eur. J. Biochem.* **9**: 21.
Zimmerman, S. B., Little, J. W., Oshinsky, C. K., and Gellert, M. (1967). *Proc. Nat. Acad. Sci. U.S.* **57**: 1841.

THE MOLECULAR ARCHITECTURE OF SYNAPTONEMAL COMPLEXES

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I. Introduction

Synaptonemal complexes are ordered structures which occur exclusively in the nuclei of gametocytes, primarily at the beginning of the

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first meiotic prophase. They form part of the paired homologous chromosomes and accompany their movements from the period in which they synapse until the moment they disjoin. Each complex is formed of two main units, or lateral elements, between which there is a layer of material apparently formed by the coparticipation of these lateral elements.

Discovery of the synaptonemal complexes (SC) with the electron microscope clarified the morphology and behavior of chromosomes at a stage (early prophase) which had been thoroughly explored with the light microscope. Whereas light microscope research provided the background to explain the cellular mechanisms of heredity, use of the electron microscope opened the way to explore the molecular basis of more specific events such as recombination and crossing over. A major contribution of this research has been precisely the discovery of synaptonemal complexes (Watson, 1952). Even though the original discovery was made at a period when resolution of the electron microscope was not fully developed, or otherwise could not be fully exploited, its importance was soon recognized and the presence of synaptonemal complexes at early prophase in both invertebrate and vertebrate spermatocytes and oocytes was expeditiously studied (Moses, 1956a, 1958; Fawcett, 1956; Sotelo and Trujillo-Cenóz, 1958; Sotelo, 1959).

Electron microscope investigations of meiotic cells have very greatly increased since 1960, and the following facts have been definitively established: (1) most of the SC's in developing gametocytes occur simultaneous with and/or represent the state of chromosome pairing (Moses, 1958; Moses and Coleman, 1964); (2) the structure of the SC's is species constant and is the same for all the members of a complement; (3) the number of SC's corresponds to the haploid number of the species; furthermore, each complex is as long as the bivalent in which it lies (Sotelo and Wettstein, 1966b; Wettstein and Sotelo, 1967); (4) formation of SC's depends on a small amount of DNA synthesis which occurs at leptonema (Hotta *et al.*, 1966; Ito and Stern, 1967; Roth and Ito, 1967); and (5) the axial frame starts to form when segments of both homologs approach each other to a distance of about 3000 Å (Moens, 1969).

Whether SC's are directly involved in genetic exchange or play a less important role is still a matter of discussion. From the investigations by several authors (Nebel and Coulon, 1962a; Coleman and Moses, 1964; Sheridan and Barrnett, 1969) we know that DNA and basic protein probably constitute a large part of these structures, but the imprecision of the methods available for detecting DNA at a substructural level makes it impossible to say with certainty whether the space separating the two main components also contains DNA. Although knowledge of the

chromosome structures related to pairing and genetic recombination has advanced, we are not yet in possession of all the information required to specify clearly the site where the macromolecules responsible for these phenomena are located.

II. Structural and Chemical Organization of Synaptonemal Complexes

A. Morphology

1. *General Description*

Watson's (1952) low magnification electron microscope investigation on rat spermatogenesis showed, in the nuclei of primary spermatocytes, pairs of dark densities which now can be recognized as identical with the ordered structures (chromosome cores) discovered by Moses (1956a,b) in crayfish spermatocytes and by Fawcett (1956) in pigeon, cat and man. Fawcett gave a three-dimensional definition of these components of meiotic prophase chromosomes which is still valid: ". . . the core appears to be a ribbon-like structure with its margins formed by two parallel dense fibrils evenly spaced on either side of a third slender linear density." The general organization of chromosome cores was confirmed shortly after by Moses (1958), this time studying the salamander *Plethodon cinereus*, and also by Sotelo and Trujillo-Cenóz (1958), who described their appearance in specimens from rat, fish, and arachnid.

The overall morphology (tripartite appearance and relationship to paired chromosomes) was described in the reports mentioned above. Later, the importance of the third linear density was emphasized by Sotelo and Trujillo-Cenóz (1960), who studied primary gametocytes of the cockroach *Blaptica dubia* and detected continuities between the filamentous components of the lateral ribbons and those of the medial one.

Moses' study on *Plethodon* allowed him to correlate the time of appearance of the so-called "cores" with the stage of chromosome pairing (synapsis), and he therefore named them "synaptinemal complexes" (now spelled "synaptonemal"; Fawcett, 1966; Wettstein and Sotelo, 1967; Smith and King, 1968; Moses, 1969). Many researchers have contributed to our knowledge of SC's, and a review of this work indicates that, although there is still much to learn about the behavior, chemical composition, and structure at the molecular level, no important variants can be expected in the SC's gross features. The following morphological data are common to all examples studied till now.

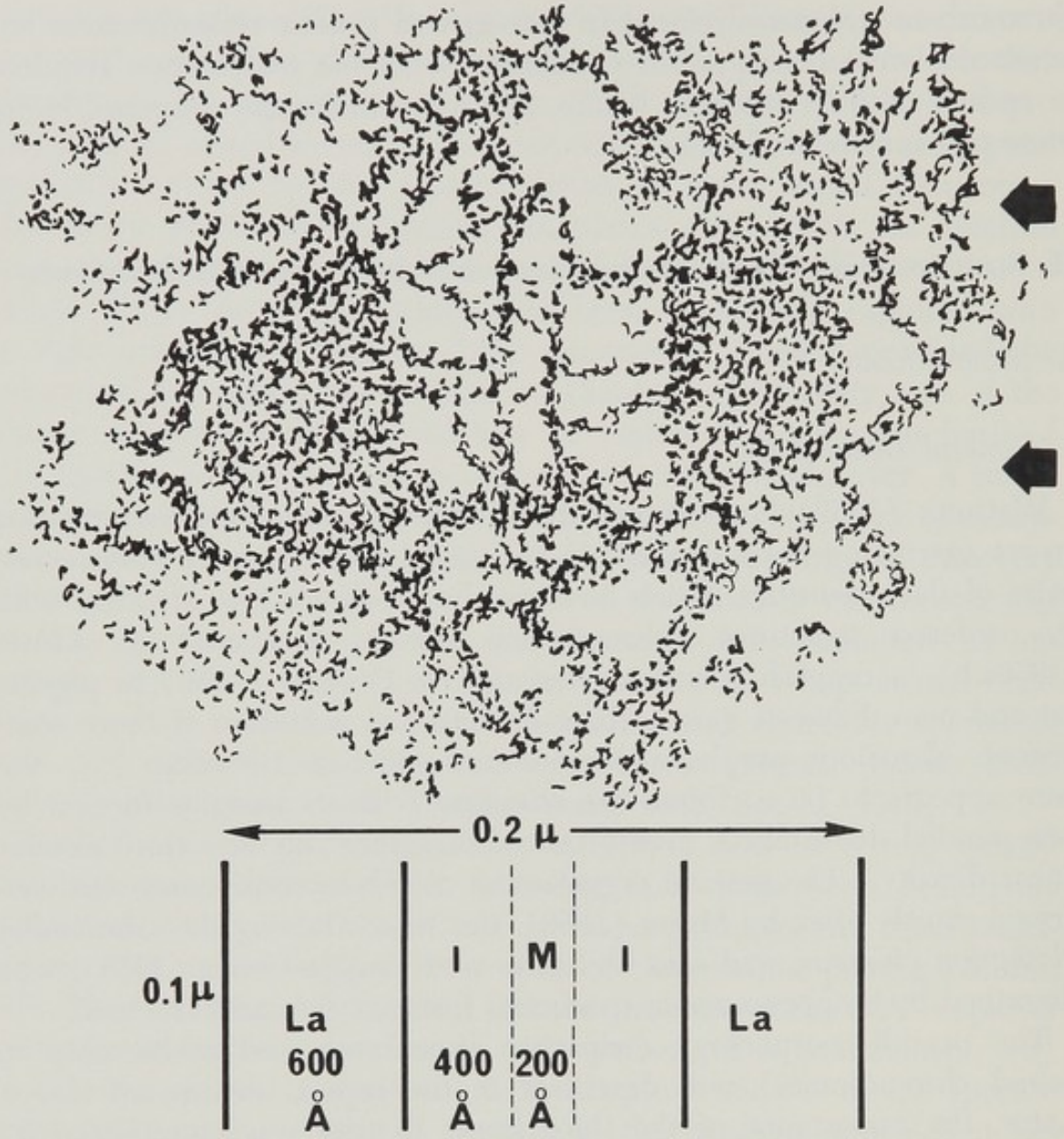


FIG. 1. Tracing of a cross section of a bivalent at early pachynema (*Blaptica dubia*, cockroach). The dimensions of the five layers composing the synaptonemal complex are shown in the linear drawing at the bottom of the picture. In addition, the figure (top and bottom) shows: (1) intermixing of fibrils from both sides of the bivalent; (2) in the intermediate space, the groups of filaments bridging the pairing space contribute to the formation of the two parallel paraxial planes indicated by broken lines below. The two thick arrows at right indicate the limits of the oval densities corresponding to the cross-sectioned lateral elements (LA). The intermediate spaces are indicated by (I) and the medial region by (M).

In cross sections, the tripartite complexes (SC's) are rectangular in outline, measuring 0.1 to 0.25 μ by 0.08 to 0.20 μ (Fig. 1). Each complex is composed of five parallel zones: two external ones, which are electron dense and compact (the lateral elements), each about 200 Å thick; two inner ones (named intermediate spaces by Sotelo and Wettstein,

1964a) which are light, loosely textured, and about 200–300 Å thick; and a medial one (sagittal) which is about 200 Å thick.

Complete views of the SC's are rarely observed in single sections unless very thick ones are examined, and obviously such thick sections impair the quality and resolution of the electron micrographs. It is seldom that the electron microscopist can record long segments lying parallel to the sectioning plane. Therefore, the best way to benefit from the electron microscope resolution is to follow the trajectory of each bivalent in serial sections (Wettstein and Sotelo, 1967; Moens, 1969). Significant data on the number and relationship of SC's with other nuclear components can be obtained if the series is long enough to allow reconstruction of whole nuclei. The procedure used to trace the bivalents and/or SC's is very simple; the first segment of an SC appearing in a series of electron micrographs is numbered and subsequently drawn marking its relative position within the nucleus. Next, corresponding segments are followed throughout the series until the whole trajectory has been reconstructed. Finally, each complex is drawn separately within a circle representing the nucleus (Fig. 2). This type of tracing is made using low magnification pictures (originals: 6000 to 10,000× enlarged up to 30,000×). For a more detailed study of individual complexes, they can be traced on a sheet of transparent paper placed over the electron micrograph; for this purpose, micrographs are taken at about 20,000× and magnified up to 100,000×.

Based mainly on this type of study, the complex illustrated in Fig. 3 has been drawn from successive micrographs taken from serial sections. It was selected as representative of SC's at synaptene-pachytene. In the upper left corner of the drawing the total length of the complex is represented as a continuous line; in the serial drawings the helical torsions of the complex and other irregularities along its trajectory can be noted.

It is evident that the two ends of the complex are attached to the nuclear envelope, and that at each point of attachment, it is surrounded by large masses of dense material; the remainder of the complex lies free in the nucleoplasm. At this stage the amorphous chromatin material of the bivalent is represented by the large masses referred to above, together with less conspicuous ones appearing disseminated along the free part of the trajectory. Some of these chromatinic bodies are united to the lateral ribbons by short or long pedicles, while the remainder are seen in direct continuity with them.

As shown in the drawings, the longitudinal continuity of the bivalent is more evident in the uniformity of the axial tripartite complex than in the paraxial chromatin. In most cases, osmium-fixed, plastic-embedded

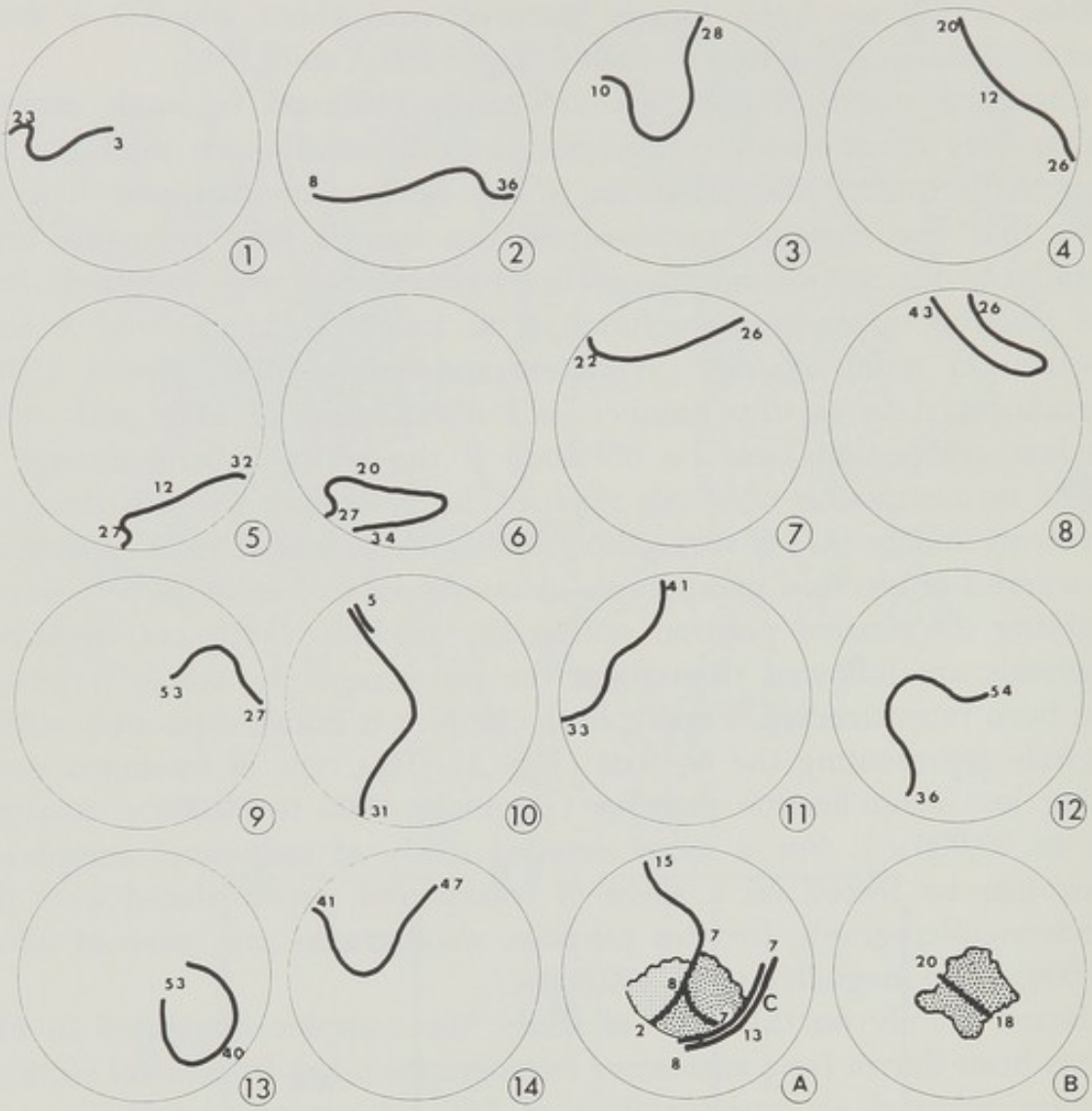


FIG. 2. This diagram represents the 14 bivalents and the supernumerary complexes involved in the associated nucleolus and sex chromosome of *Gryllus argentinus* spermatocytes. In each circle the trajectory of one bivalent has been schematized; the figures at each extremity of the bivalent represent the first and last section numbers in which each one was seen, and their relative positions within the nucleus. For instance, bivalent 1 was traced in 20 sections and bivalent 2 in 28. The figures placed along the trajectory indicate either that the extremities or an intermediate segment appeared first in the consecutive sections. Bivalent 5 first appears in section 12, whereas the extremities go down to sections 27 and 32, respectively. Bivalent 10 shows a short supernumerary segment starting at the nuclear membrane. The chromosomes have been numbered according to the order in which they appeared in the series. Circles A and B represent the nucleolus, the sex chromosome and the supernumerary synaptonemal-like complexes involved in the association. (A) The supernumerary elements originating from two merging groups (one nucleolar, the other interrelating both bodies) and running up to the nuclear membrane from section 2 and 7 up to section 15. (B) One unit running inside a nucleolar channel. (Ac) The extension and position of the tubular complex. In this circle the chromosome and nuclear bodies are not represented. (From Wettstein and Sotelo, 1967.)

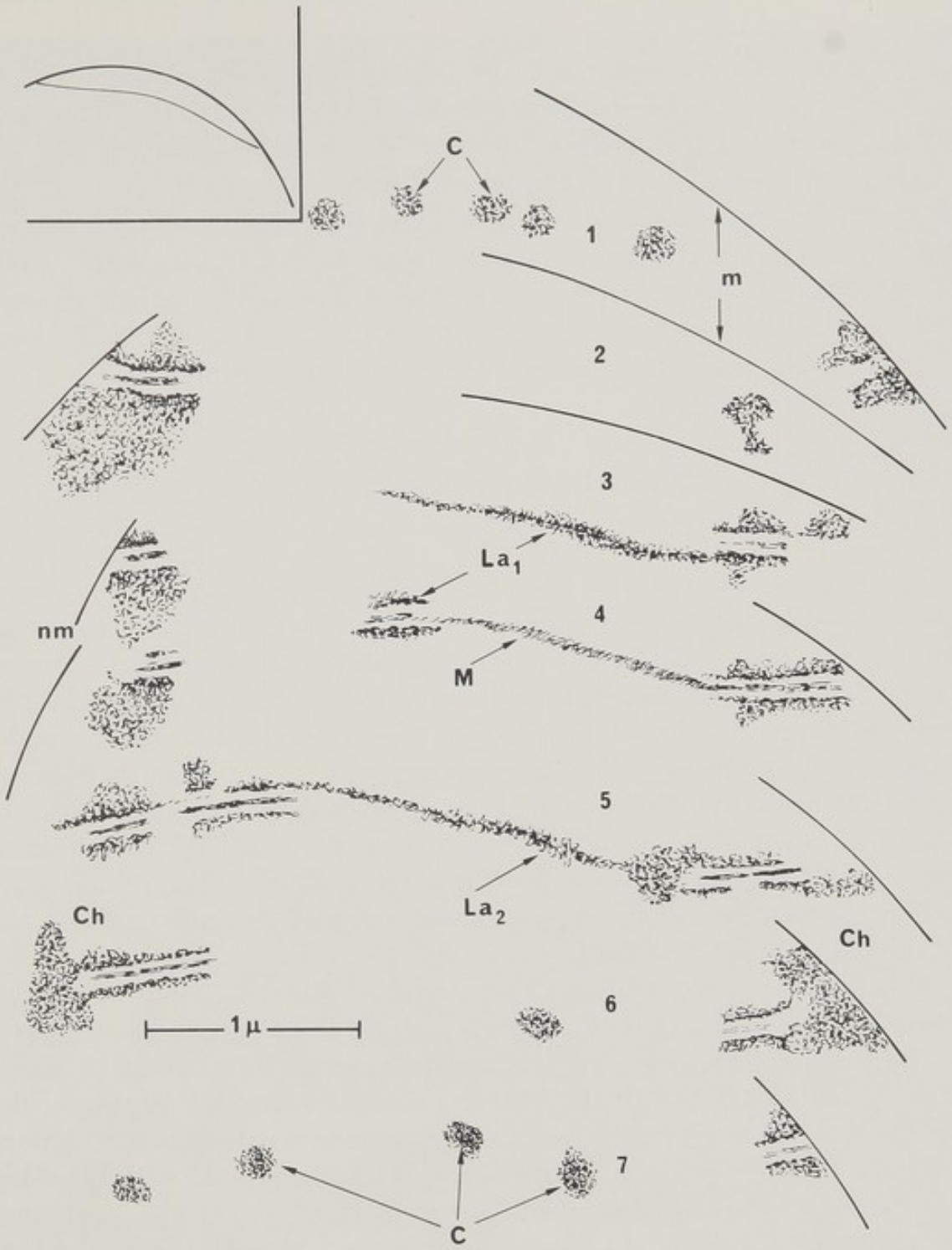


FIG. 3. Tracing of seven serial sections of a bivalent at zygonema from *Gryllus argentinus*. Number 1 shows a row of five chromomere-like masses located along the bivalent but at some distance from the synaptonemal complex, together with a larger mass contacting the nuclear membrane (m) and forming part of the material seen at section 6 marked Ch (for chromocenter); (2) shows at left, one extremity of the synaptonemal complex attached to the nuclear membrane (nm); (3) shows one lateral element (LA_1); (4) shows the medial component and (5) the other lateral element; (6) and (7) show the two extremities and another row of chromomere-like masses. In the upper left corner the entire trajectory of the synaptonemal complex in relation to the nuclear outline is represented. Magnification before reduction 48,000×. Total length of the complex was 6 μ.

early prophase nuclei appear to be largely homogeneous, and chromosomes cannot be identified except where linear dense elements have already formed. Dispersion in the nuclear sap and uniform stainability of the DNA–protein complex may account for this condition. Examples of synaptene chromosomes showing a more definite chromatin coating have been reported (Moens, 1968; Sotelo and Trujillo-Cenóz, 1960). Despite the apparent isolation from the milieu described above, continuity of components between the dense part (ribbons) and the loose network of the adjacent chromatin occurs at a level below 100 Å.

2. *Structure of the Parallel Lateral Elements and the Peripheral Chromatin of the Bivalent*

Examination of adjacent thick and thin sections with the light and electron microscope (later supplemented with cytochemical tests, to be reviewed below) led Moses (1956a,b) to state that the lateral elements of the SC's are integral with the adjacent field of chromatin. This concept was subsequently shared by most of the researchers working on this subject, but as Moses (1968) later pointed out ". . . continuity between the microfibrils and filaments of the complexes and those of the chromosomes is not sufficient proof of their identity." The high resolution of the electron microscope has been frustrated by the lack of adequate techniques for identifying specific molecules both in the complexes and in the field surrounding them.

Whether loose or compact, the material surrounding each SC appears to be formed by intermingled fibrils, which at low magnification seem to be independent units. In longitudinal views of the bivalent, fibrils coming into contact with the lateral ribbons are roughly perpendicular to the axis, thus giving a hairlike appearance to their outer borders. In some species the laterally projecting fibrils are spaced with a ladderlike periodicity. This arrangement seems to exist even in the axial cores of leptotene threads (*Lillium longiflorum*, Moens, 1968; *Locusta migratoria*, Moens, 1969; *Scaptericus borreli*, Sotelo and Wettstein, unpublished).

At higher magnification and resolution, it is seen that the fibrils are integrated by ropelike, braided filamentous units less than 25 Å thick (Fig. 4). Comparison of chromosome material from different origins, either mitotic or meiotic, shows that this structure consistently occurs (cf. Solari, 1964, Fig. 4a and b; Sotelo and Wettstein, 1965, and Wettstein and Sotelo, 1965a). These fibrillar chromosomal units have been described in most papers dealing with chromosome ultrastructure; how-

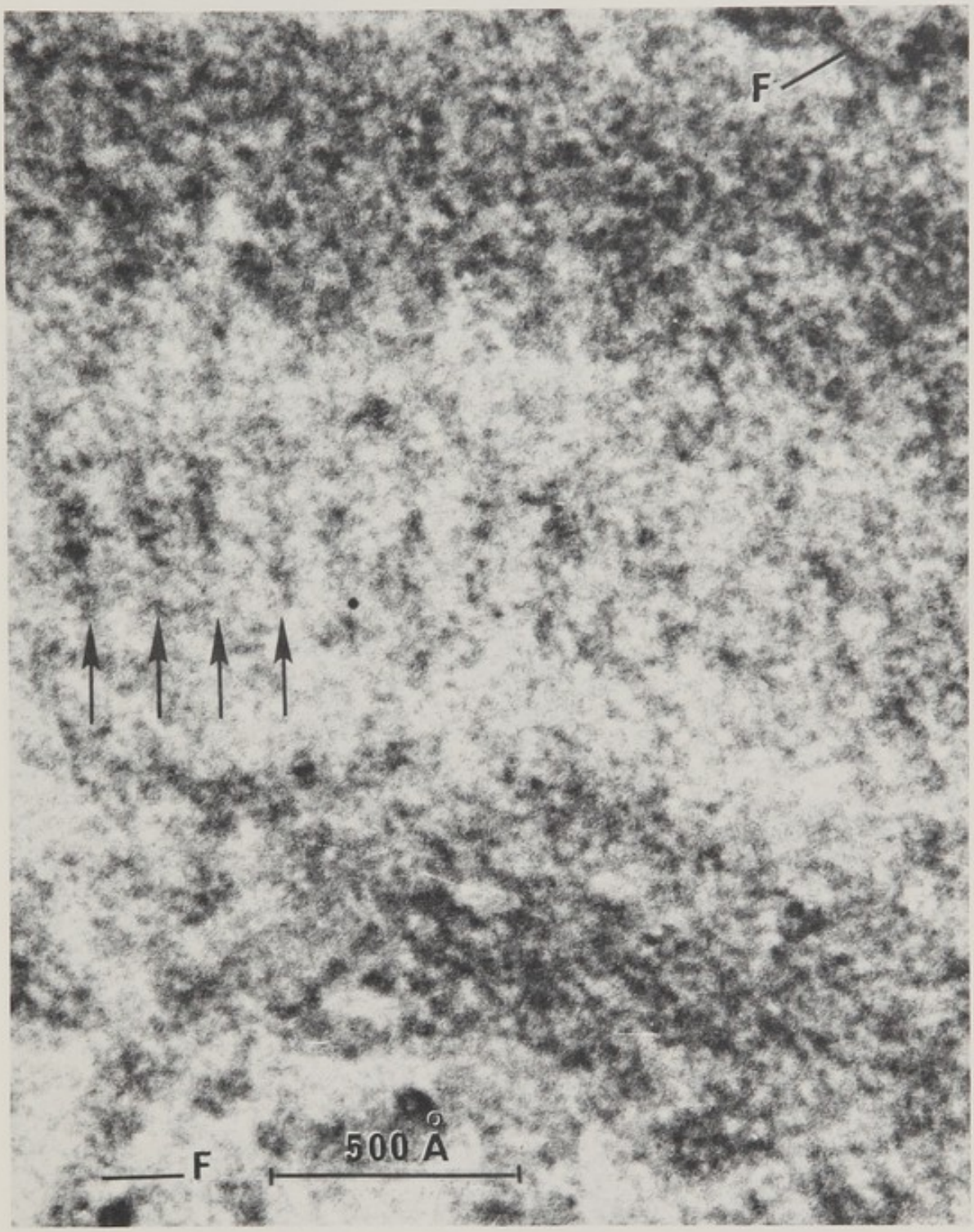
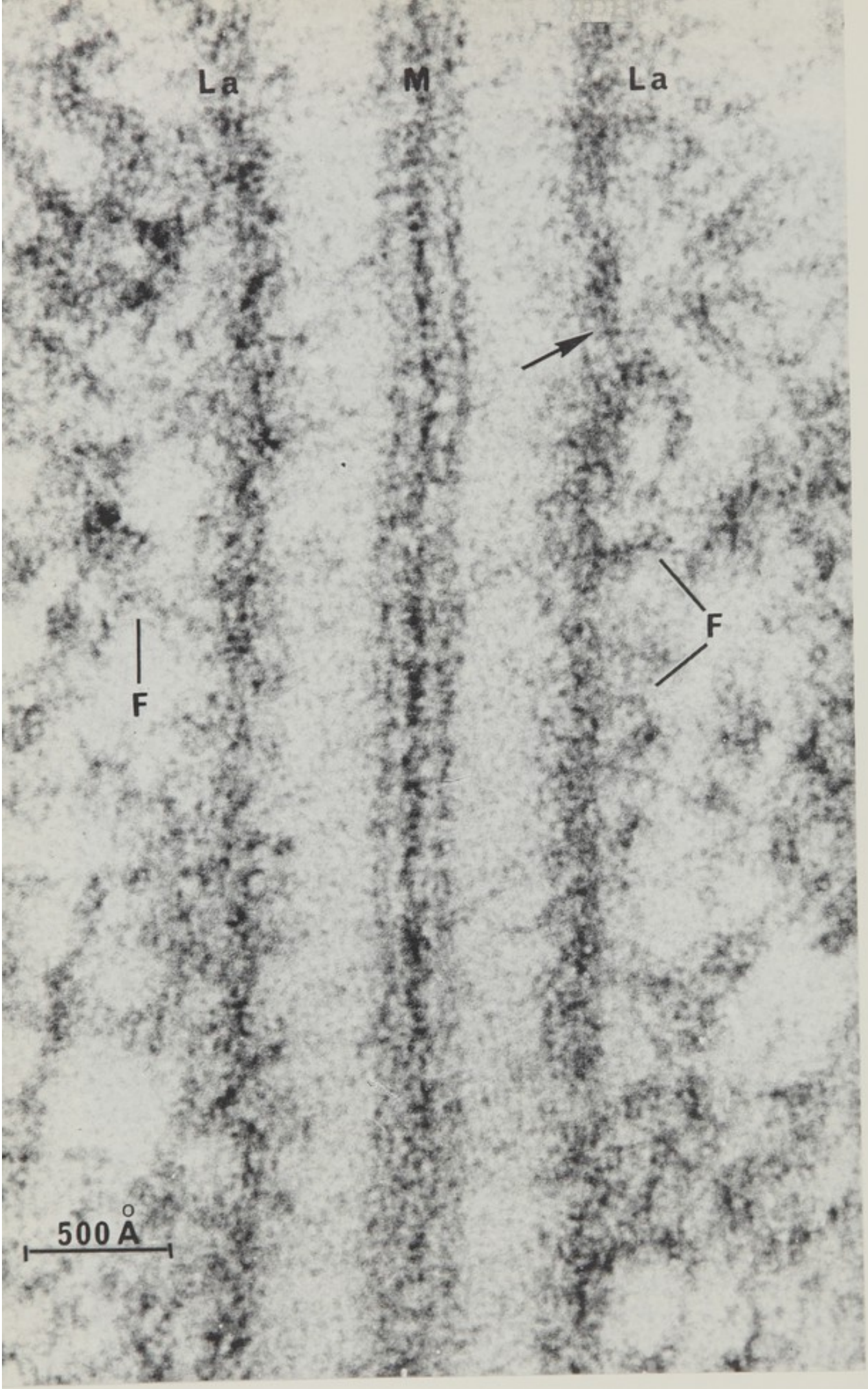


FIG. 4. In Acrididae, the pairing space of a synaptonemal complex is crossed by filaments or groups of filaments which form evenly spaced transverse units. Each unit contains an undetermined number of filaments which cannot be counted because they do not follow straight trajectories. This segment of pachytene SC has been trimmed from a longer longitudinal oblique view taken at a place where the complex rotates 90°. Therefore only some of the transverse units are clearly depicted (arrows). The curved lateral elements of the complex are seen at the top and bottom of the picture. Some of the chromatin fibrils from the field surrounding the bivalent can be seen joining the lateral elements at (F). *Laplatacris dispar*, 600,000×.


La
M
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ever, the indiscriminate use of terms such as threads, fibrils, microfibrils, or filaments, evidently designating the same components has introduced considerable confusion, particularly considering that their diameters have been measured in many instances from low magnification pictures. In this article the term *thread* is reserved for major units visible with the light microscope, *fibrils* for composite units about 100 Å thick, and *filaments* for single units less than 25 Å thick. Single units thicker than 25 Å, as well as composite fibrils less than 70 Å, generally are not observed in chromosomes. Moses (1964) some years ago described the possible causes of error in estimating thickness. After mentioning a range of about 100–500 Å he concluded that, "Despite the many sources of variation, it is now a fairly consistent observation that a twisted microfibril of the order of 100 Å in diameter is a regular component of chromatin."

Dimensions observed in chromosome fibrils prepared by the air-water interface spreading method (Gall, 1963) differ only slightly from the dimensions obtained by thin sectioning of plastic-embedded material. Recent papers indicate that inactive chromatin appears as a network of fibers which are consistently 200–250 Å thick (Ris, 1967). Whole mounted human metaphase chromosomes contain fibers ranging from 100 to 500 Å in diameter (DuPraw and Bahr, 1969).

Both the dimensions and morphology of fibrils referred to in this article have been determined from sections thinner than 300 Å cut from osmium-fixed araldite-embedded specimens (electron micrographs taken at original magnifications of 40,000–60,000× were enlarged up to 400,000–600,000×). At these magnifications, it can be seen that the 25 Å filaments are not confined to the limits of each fibril, but may pass from one to another constituting loose or compact networks (Wettstein and Sotelo, 1965a).

The transition zone between the SC lateral ribbons and the peripheral field of chromatin is ill-defined (Fig. 4). In general, the fibrils dissociate entirely into their elementary components and these become packed within the ribbon without showing any evidence of order (Fig. 5).

FIG. 5. Frontal longitudinal view of a synaptonemal complex in which the trilayered structure of the medial component (M) is particularly obvious. In the places marked (F) continuity between the elements composing the lateral ribbons (La) and those forming the 100-Å fibrils of the adjacent chromatin is also seen. Bridges between the lateral elements and the medial component are less apparent in frontal than in oblique sections, particularly if the thickness of the section is below 400 Å; however, a few can be observed. Some of the 15–20 Å filaments which are the basic morphological units of the chromosome complexes are marked by an arrow. *Gryllus argentinus*, 420,000×. (From Wettstein and Sotelo, 1967.)

A few exceptions have been noted in this respect; for instance: (1) the squarelike pattern found in the grasshopper *Laplatacris dispar* (Sotelo and Wettstein, 1965; cf. Moens, 1969), which is probably similar to the linear pattern reported by Moses and Coleman (1964) in rat synaptonemal complexes; (2) the netlike disposition found in the cockroach *Periplaneta* (Sotelo and Wettstein, 1966a); and (3) the transverse ordering of components observed by Westergaard and von Wettstein (1966) along the ribbon's inner part in the fungus *Neotiella rutilans* (Fig. 6).

Modern data on DNA content prior to pairing indicate that each homolog has already duplicated before the onset of prophase (Taylor,

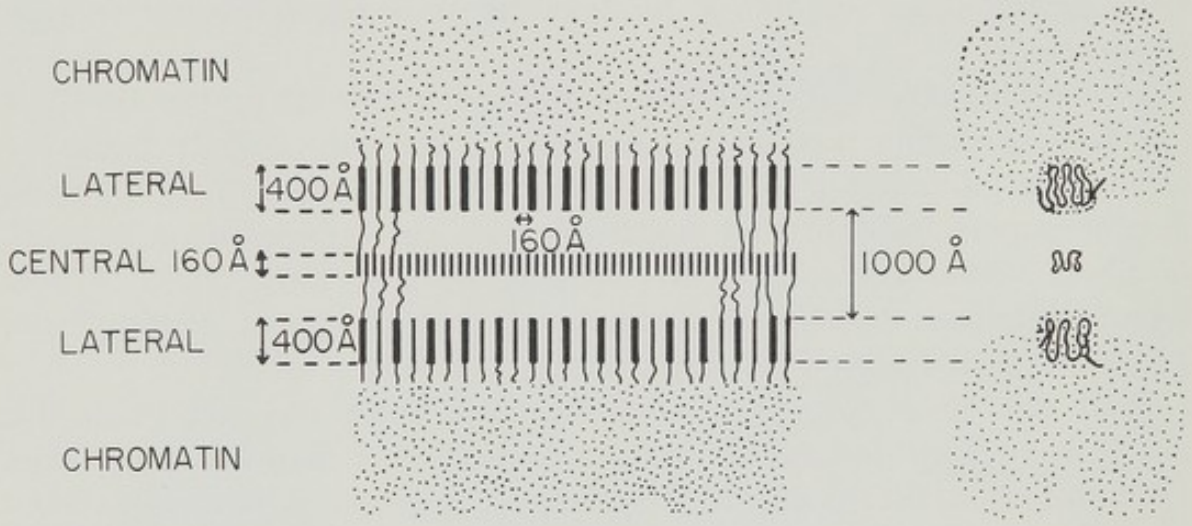


FIG. 6. Diagram of a synaptonemal complex in *Neotiella*. (From Westergaard and von Wettstein, 1968.)

1965); consequently at the stage of synapsis each bivalent is already composed of four chromatids. This fact has led several researchers to search for a bipartite structure in the SC ribbons. Nebel and Hackett (1961b) were the first to consider twin, ill-defined longitudinal densities within the lateral elements as representing sister chromatids. Later, Woollam and Ford (1964) reported that each lateral element is composed of an inner and an outer lamina, and they suggested that according to the concept of Taylor *et al.* (1957) the inner one would correspond to a new chromatid, whereas the outer one would be an old chromatid. When visible, both units may be helically twisted (Baker and Franchi, 1967a; Sotelo, 1969), but in most cases the compactness of the lateral ribbons impairs discrimination of individual densities within their substance. The helical double structure of the lateral elements would be in agreement with the classic spiral concept of chromosome structure.

In fact, the helical disposition of the SC ribbons and the double helical structure of DNA are the best examples of this spiral concept at an ultrastructural level.

Available evidence suggests that two chromatids or parts of them may be represented in the thickness of each lateral element, but only in some cases is it possible to recognize a bipartite structure. Coarseness of fixation plus extraction of soluble macromolecular material may favor visibility in the electron microscope.

3. *The Lampbrush-Like State*

In the female germ cells of most mammals, the meiotic chromosomes can be detected with both the light and the electron microscopes until the end of pachytene when they disperse in the nucleus and become indistinguishable. This dispersed-chromosome state more or less coincides with the so-called major period of growth, and ends when the nucleus reorganizes for the first meiotic division. In contrast to mammals, the chromosomes of many lower vertebrates (fish, amphibians, reptiles, and birds) remain visible after pachytene, acquiring a configuration known as a lampbrush structure (Rückert, 1892).

In some mammals (human and monkey), the chromosomes of the primary oocytes remain at a diplotene stage and bear faint lateral projections. Baker and Franchi (1967b) reviewed these data, and used the electron microscope to study the chromosomes of human primary oocytes at this stage; they found granular clusters located about 1 μ from the chromosome axis and united to the latter by looplike fibrillar strands. The authors traced several of them at low magnification and concluded that the loops are extensions of the longitudinal axial core (see Section IV,B). It is interesting to note that, at the stage studied by Baker and Franchi, the chromosomes still retain axial cores which may be the remnants of the SC lateral elements from the preceding prophase stages.

In amphibians, the lampbrush chromosomes attain a considerable length without losing their individuality, continuity, or the paired relationships established at preceding stages (chiasmata). Their loops are readily seen with the light microscope. By contrast, in mammals as noted previously, a looplike organization is not clearly discernible with the light microscope, and detection of lateral projections is difficult even with the electron microscope; in some cases, these may have been artificially created. Full consideration of this matter is beyond the scope of this chapter, but it is brought to the reader's attention because several authors have postulated a similar structural organization for meiotic

chromosomes from different species, i.e., male meiocytes, involving synaptonemal complexes as a structural part of the lampbrush organization (Nebel and Coulon, 1962a; Moens, 1968).

The modern concept (Gall, 1956, 1963) is that each lampbrush homolog is formed by two chromatids, each constructed like a chain or rosary of minute chromomeres longitudinally connected by the interchromomeric fibers which as a whole (duplex) have been estimated to be from 75 to 200 Å thick (Miller, 1965). At the chromomere level, the chromatids would spin out to form loops on either side of the axis. Experimental tests on lampbrush chromosomes prepared by a surface spreading procedure have provided evidence that each loop is formed by an axial thread (the chromatid) sensitive to DNase and a coat or matrix sensitive to proteases and RNase (Miller, 1965). Paired loops arising from the same chromomere are similar in extension and morphology (i.e., symmetrical), but there are considerable differences in morphology between pairs of loops arising from different loci on the chromosome. Furthermore, the ribonucleoprotein coat of each loop is distributed unevenly in such a way that each loop has a thin and a thick extremity.

Nebel and Hackett (1961a) were the first to look for a similarity between the ultrastructure of spermatocyte chromosomes at pachytene and the lampbrush organization referred to above. They interpreted as lampbrush loops the 100 Å fibrils which emerge hairlike from the SC lateral elements toward the surrounding chromatin fields; however, they made clear that these fibrils could not be traced from end to end. In a more detailed study of pigeon spermatocytes, Nebel and Coulon (1962a) tried to provide new evidence for a lampbrush organization; their hypothesis began with the assumption that there are two sister chromatids in the thickness of each SC lateral element. Consequently, they recognized two kinds of loops, those that are sisters since they arise from both chromatids at the same level and those that are not because they are attached at different levels. These loops supposedly arise from the inner layer of the lateral elements.

As described previously, many authors have recognized fibrillar projections emerging laterally from the SC's, and also the impossibility of following their trajectories and relationships in thin sections (Moses, 1968). Furthermore, the oocyte lampbrush structure is an adaptation for the special functional requirements of the developing egg, which probably should not be compared with those of male meiocytes. In the smaller nuclei of the latter cells, the long DNA molecules are probably multifolded, and such folds may possibly present the characteristics of loops. Nevertheless, it does not seem likely that the organization of oocytes and spermatocytes are entirely comparable.

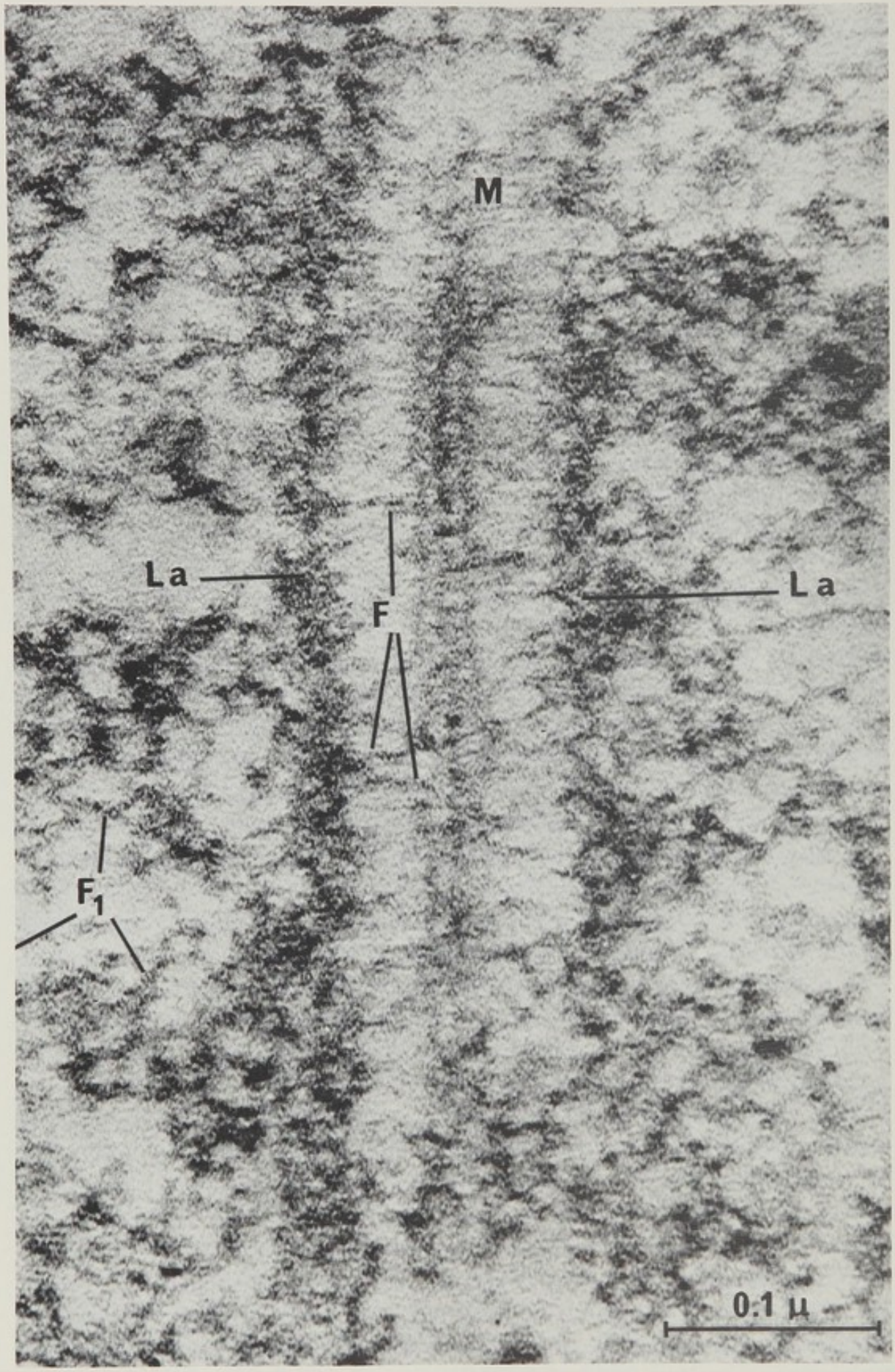
4. *Structure of the Pairing Space and the Medial Ribbon*

It is well established that the two SC lateral elements do not fuse or meet along their lengths. It would follow that genetic exchange must occur either in the space between the two ribbons or in the chromatin surrounding them. The existence of a system of filaments which cross like bridges from one lateral element to the other has been considered as favoring the first possibility. Because the existence of a medial ribbon was at first considered variable, the attention of researchers was concentrated on the structure of the lateral elements and on their relation to the chromatin. In 1960, Sotelo and Trujillo-Cenóz found that the components of the lateral ribbons are continuous with filaments lying in the intersynaptic space. Later, Meyer (1964), after discovering that achiasmate male meiocytes of *Drosophila* do not have SC's, maintained that in female meiocytes the number of crossing filaments parallels the number of alleles. Subsequently, crossing filaments and medial ribbons gained more attention from researchers, and their organization was clarified by high resolution studies (Sotelo and Wettstein, 1964a, 1966a,b).

The SC lateral elements are interconnected by numerous groups of filaments (or single filaments 15–25 Å thick) which cross the pairing space transverse to the main longitudinal axis (Fig. 7). In a number of species, these crossing filaments meet along the sagittal plane and contribute to the formation of a medial ribbon; in other species, crossing occurs but no definite medial ribbon is formed. Synaptonemal complexes can be classified into three main groups according to the complexity developed by this system. The first group (referred to in this article as group A) includes those SC's which show only a disordered crossing of filaments; in the second group (group B), crossing is characterized by the formation of a sagittal density in which the filaments intermingle in a disordered way (Fig. 8); and finally the third group (group C) includes those SC's that show medial ribbons organized according to a precise structural pattern (Figs. 4, 5, 7, and 9).

SC's belonging to group A show very simple features; the bridging structure is made up of thin filaments not easily detected at low magnifications. So far few examples of this type have been encountered (*Acanthopachyllus aculeatus*, Opilion, Wettstein and Sotelo, 1965b; *Pheteremia fetida*, earthworm, Sotelo, unpublished).

SC's belonging to groups B and C are frequently found in animal and plant species. In group C two types of array or structural pattern have been encountered; (a) those in which crossing occurs only in a transverse direction; and (b) those in which the crossing filaments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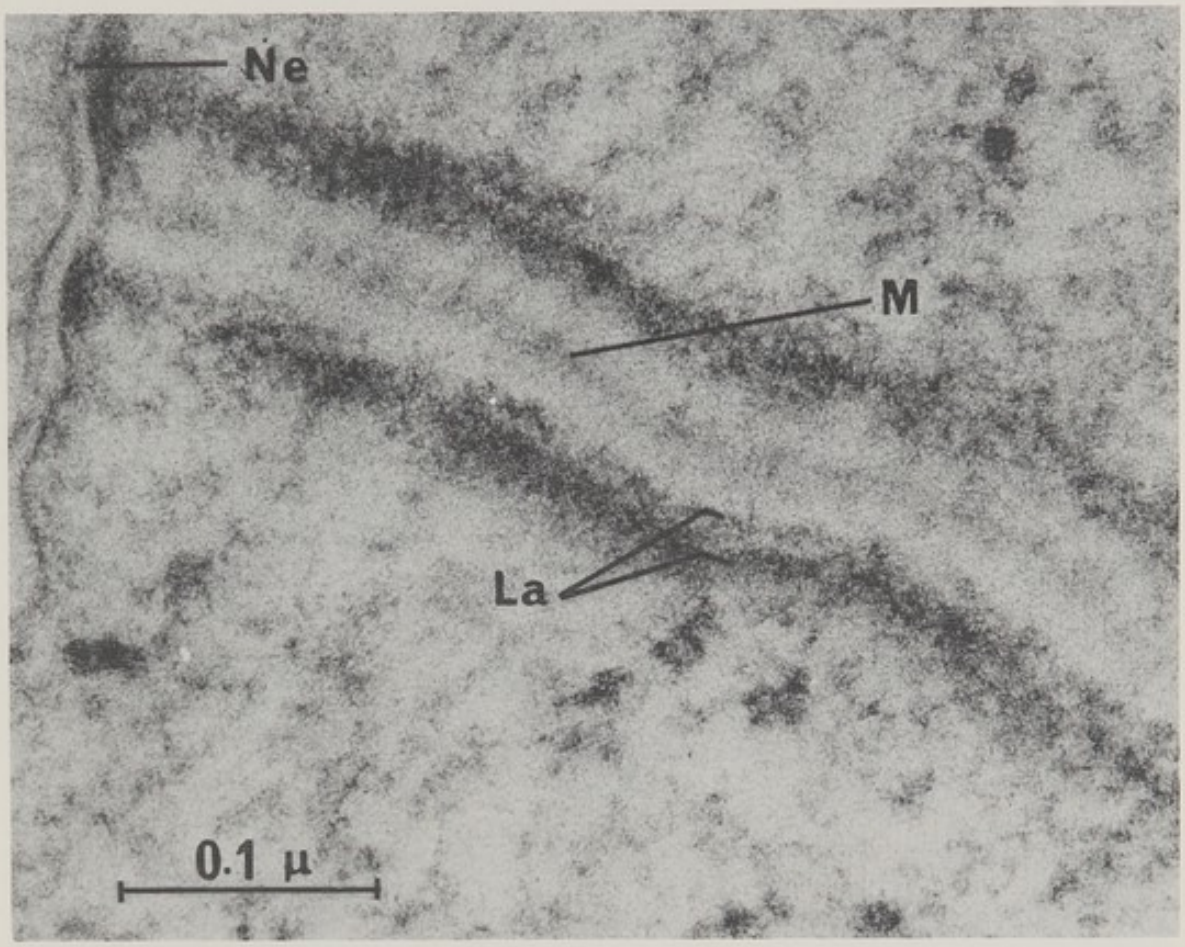


FIG. 8. In mammals the medial ribbon of a synaptonemal complex has an orderly structure in some species, but in others no structural pattern is observed. The SC seen in this figure was prepared from a dog spermatocyte. The medial ribbon (M) shows no definite organization, at least at this early stage (synaptene-pachytene). The complex is attached to the nuclear envelope (Ne) and is frontal to the observer. The cleavage into two layers of the lateral element (La) may be regarded as an unexplained detail. 130,000×.

change direction to form longitudinal planes parallel to the inner borders of the lateral ribbons. The structural type of group C(a) has been particularly observed in Acrididae (Fig. 4). In species of this order, Sotelo and Wettstein (1965, 1966a) found that the crossing filaments arise from the inner face of the lateral elements and assemble in regularly spaced flattened bundles, which are somewhat loosely arranged in the

FIG. 7. An oblique longitudinal view of a synaptonemal complex, which illustrates better than frontal section (compare with Fig. 5) the components crossing the intermediate space and joining the lateral elements with the medial ribbon (M). At this magnification, the most clearly depicted are the filaments marked (F). The elements are slightly distorted by movement of the specimen during photographic exposure; (La) lateral elements; (M) the three planes of the medial ribbon; (F_1) 100 Å fibrils of the chromatin surrounding the complex. Mid-pachytene; *Gryllus argentinus*, 280,000×.

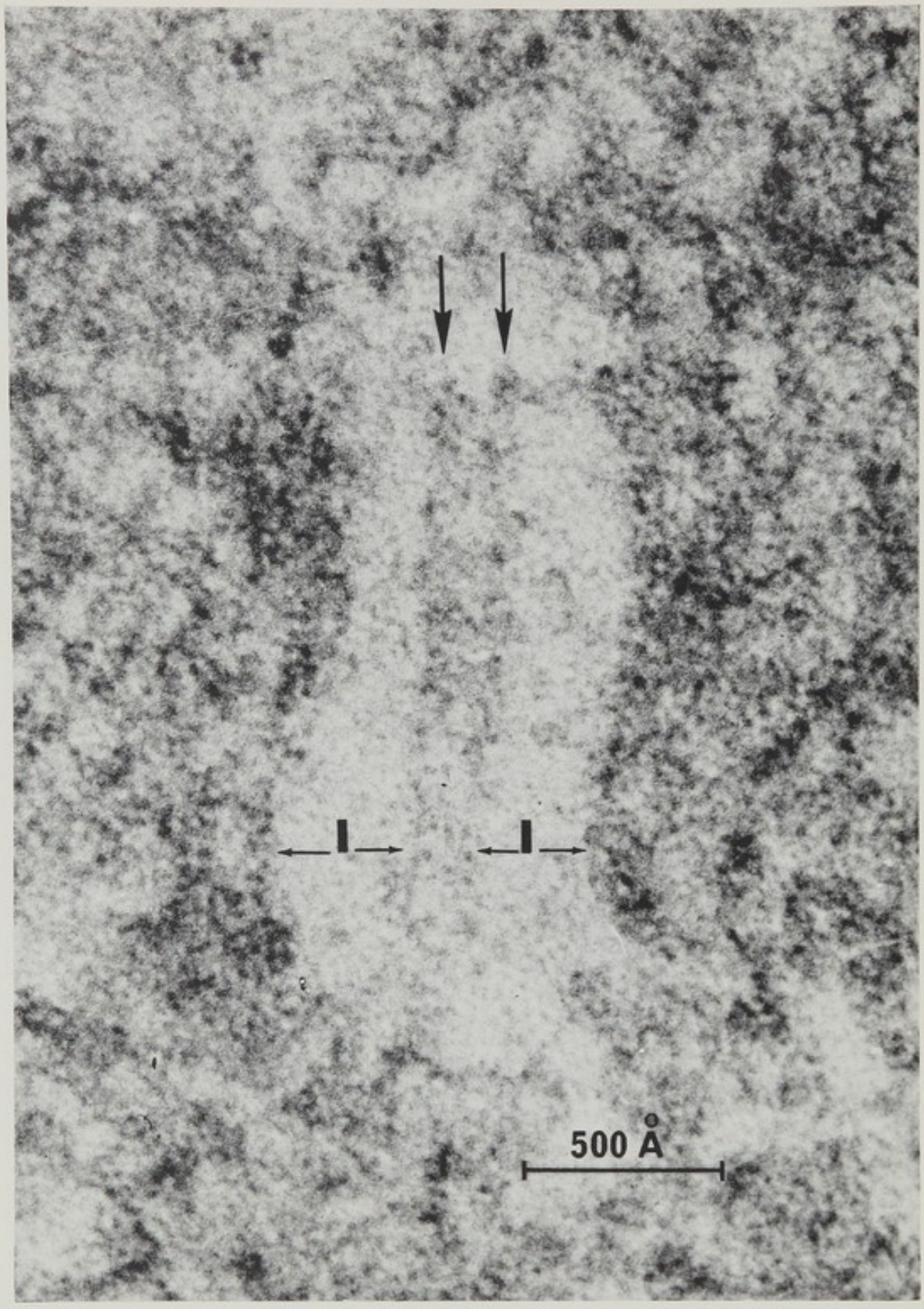


FIG. 9. In cockroaches, the cross-connecting filaments traverse the intermediate space (I) obliquely; for this reason they can scarcely be seen in frontal views like this one, and they form two parasagittal longitudinal planes (arrows). The peripheral chromatin and the lateral elements show the same netlike structure, to such an extent that in sections thinner than 300 Å the latter are not easily recognized. Intermixture of fibrils from both sides is obvious at the top and bottom of the picture (compare with Fig. 1). Early pachytene, *Blaptica dubia*, 500,000×.

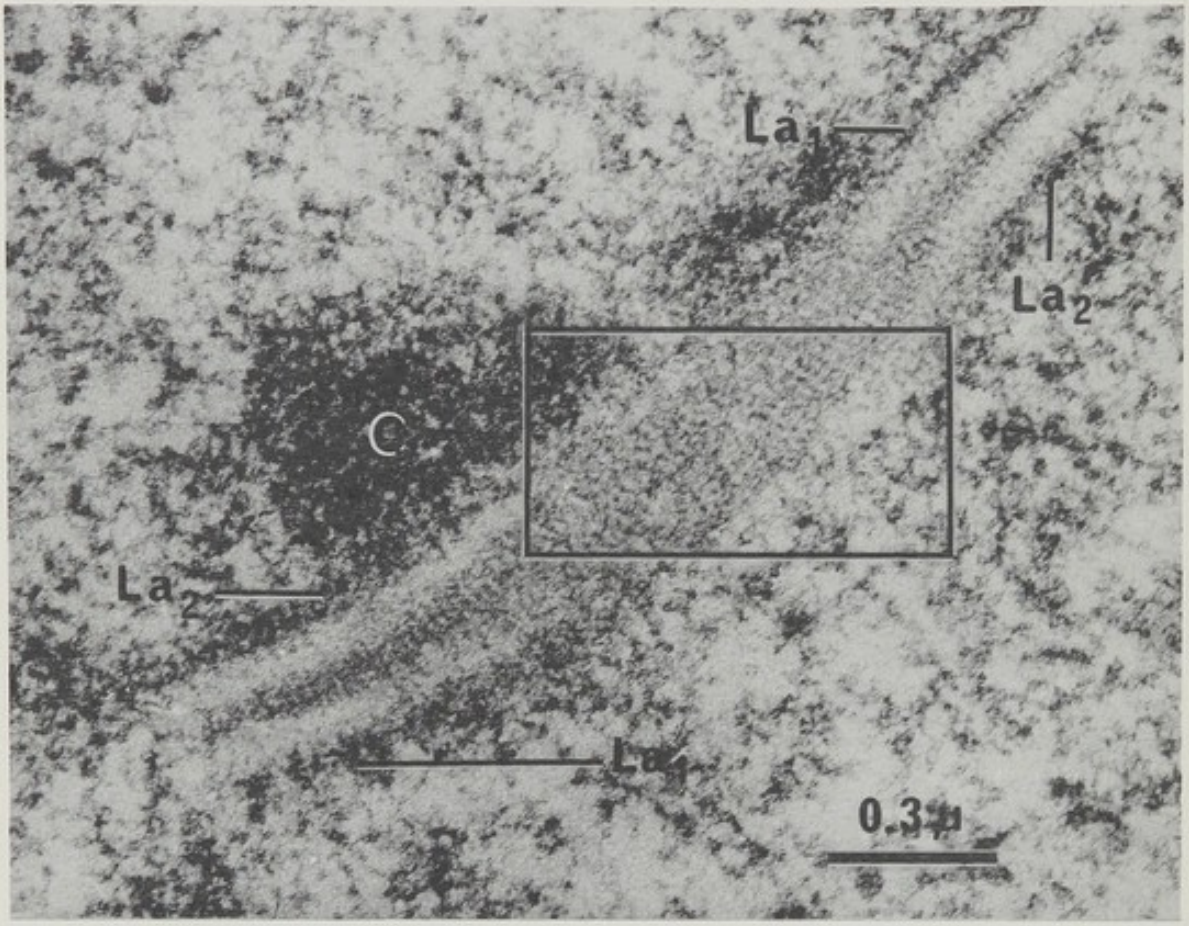
intermediate space though packed in the sagittal region to form a distinctive medial density.

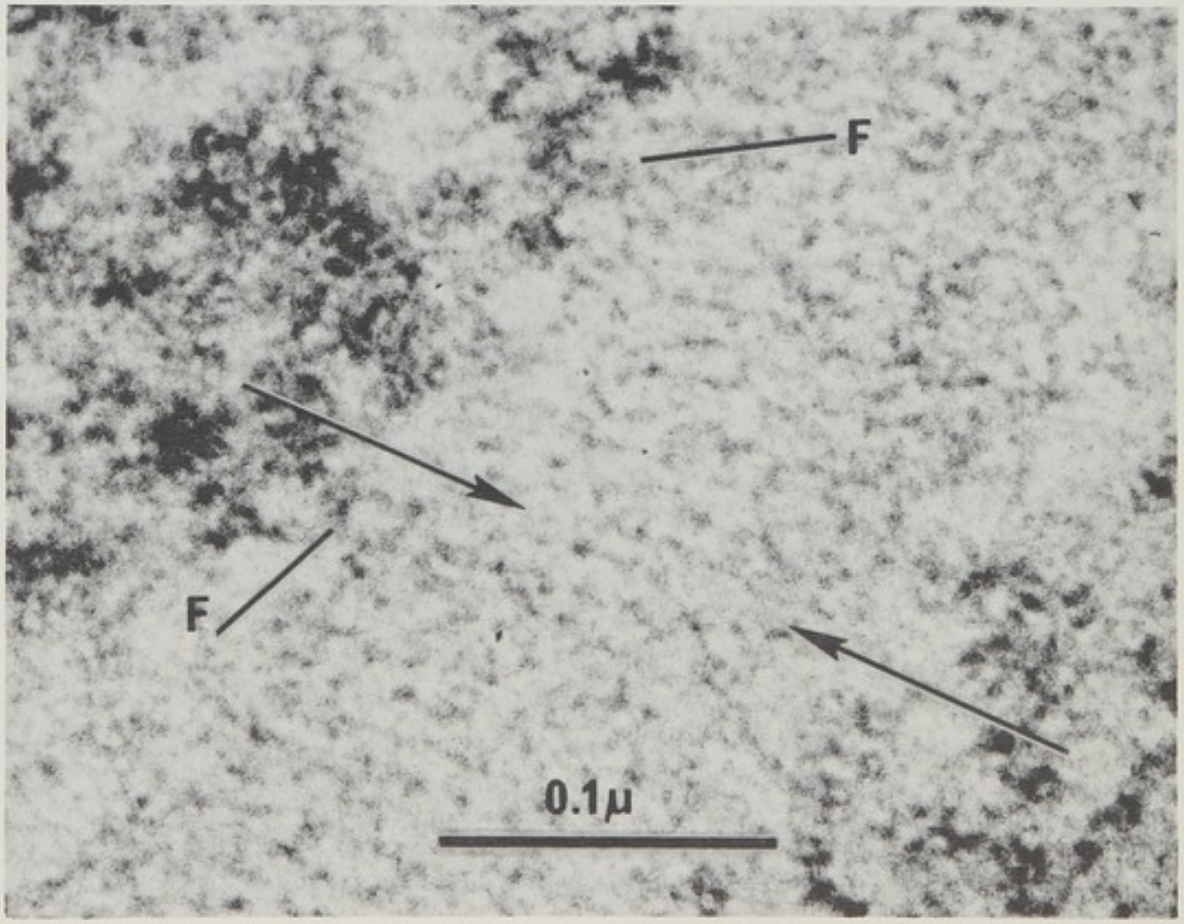
The structure of the pairing space in the fungus *Neotiella rutilans,* which was described by Westergaard and von Wettstein (1966), is comparable to the Acrididae pattern. In *Neotiella,* the inner layer of each lateral ribbon is transversely striated and the crossing filaments emerge from the striae. These filaments intermingle transversely along the sagittal plane, evidently following an ordered pattern not clearly discernible in the electron micrograph illustrating the article. A schematic interpretation by the authors is shown in Fig. 6.

The key characteristic of structural plan C(b) is the subdivision of the medial ribbon into two or more parallel longitudinal planes or layers (Figs. 1, 5, and 9). At low magnification, frontal views of the SC's show each plane as a continuous line, but the components of the planes (or lines) can be resolved separately at higher magnification. Within each layer, filaments from the bridging or crossing system incorporate into the immediate layer and then orient in any of the following three directions: longitudinal, transverse, or anteroposterior. The longitudinal segments contribute to the linear appearance mentioned above. Transverse segments may cross from one layer to another, making the medial ribbon appear scalariform in frontal views (cf. Guénin, 1965; Guénin and Gautier, 1960). Anteroposterior segments are best depicted in lateral views of the medial space (Figs. 10 and 11); in many cases they form regularly spaced bundles which at low magnification are seen as striae ($1\,\mu = 100$ striae). In summary, the medial ribbon is a three-dimensional lattice composed of filaments 15–25 Å thick which may run singly or associated into thin bundles. For a schematic interpretation of this lattice see Sotelo (1969).

Exact measurement of the true thickness of filaments is difficult and depends on such factors as the state of preservation of the material, precision of focus, and final magnification of the picture. Through-focus series reveal a considerable variation in apparent thickness between close-to-focus micrographs and underfocused ones. However, on account of the better contrast, it is generally preferable to show the filaments in slightly underfocused pictures. It is understood that defective conditions inherent in the microscope or in the specimen, such as astigmatism or thermal expansion, have been previously eliminated.

The number of longitudinal layers is variable. Double- and three-layered patterns, which are common among insects, were first studied in detail in cockroaches and crickets (Sotelo and Trujillo-Cenóz, 1960; Sotelo and Wettstein, 1966a). Although we have not observed more than three layers, the mosquito *Aedes egipti* described by Roth (1966)


La1
La2
C
La2
La1
0.3μ


F
F
0.1μ

may be an exception to this rule. Each species shows a characteristic pattern which does not change from one specimen to the next. Moreover, long-term studies on *Gryllus argentinus* (about 25 generations followed in this laboratory) have established that no structural variation occurs through successive generations. Occasionally a pattern found in several species of one family may be encountered in species belonging to a different family or order; for instance, the cockroach pattern (Figs. 1 and 9) is similar to the one found in Mantidae (Sotelo, 1969) and in Coleoptera (Gassner, 1969).

B. Cytotopochemistry

Nebel and Coulon (1962b) studied the action of DNase, RNase, and trypsin (alone or combined) on pigeon testis fixed in formol, osmified, and embedded in Epon. They reported the following results: (1) Buffer and water treatment (controls) do not significantly affect the organization of SC's or their surroundings; (2) DNase (2 hours treatment) causes a nearly complete loss of the lateral loops of the chromosomes; however, the lateral and medial SC components do not show changes; (3) RNase (4 hours treatment) causes chromosome condensation and loss of material in the interchromosomic surfaces, but the organization of SC's is not essentially affected; the ribosome-like granules of the nuclear gap disappear after treatment; (4) after trypsin treatment, the chromosomes appear shrunken and all intranuclear structures except chromosomes seem washed out. The SC's look coarser and fibrous, but are essentially preserved; (5) combined treatment tends to destroy all nuclear structure completely, but small segments of SC's are found preserved.

Coleman and Moses (1964) fixed rooster spermatocytes with glutar-

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Figs. 10 (*top*) and 11 (*bottom*). When the plane of section coincides with the anteroposterior or sagittal planes of a synaptonemal complex, the pattern of the subunits forming the medial lattice can be analyzed. In Fig. 10 the SC rotates 180°, and therefore the two ends are nearly frontal to the observer whereas the middle segment is seen laterally. The sectioning knife has taken away a large part of the helically twisting lateral elements, identified (La_1) and (La_2). A chromomere-like mass of fibrils is seen at the center of the picture. In Fig. 11 the morphology and relationships of the filaments (F) composing the medial layers in the same species is shown in a section thinner than the one in Fig. 10. This particular field was selected because the plane of section has the same orientation as seen in the rectangle drawn over Fig. 10. Both pictures are from *Gryllus argentinus* spermatocytes at early pachytene. Fig. 10, 48,000×; Fig. 11, 320,000×.

aldehyde, digested the tissues with DNase until nuclei were no longer Feulgen positive, and postfixed with osmium tetroxide. Sections from araldite-embedded samples were stained with uranyl acetate, indium trichloride, and lead hydroxide. They found that (1) in glutaraldehyde and osmium-fixed material stained with lead or uranyl all parts of the SC's are visible; (2) with indium alone, the microfibrils and inner layer of the lateral ribbons stain positively, the outer layers stain weakly, and the medial ribbon does not stain; (3) in DNase-digested, indium-stained samples, no staining is visible; (4) in DNase-digested sections with indium and other heavy metal staining, all parts are visible; (5) increase of contrast by reprinting of electron micrographs showed that in indium-stained samples thin filaments do cross the pairing space; the authors cannot exclude that these filaments may correspond to DNA-containing material.

Sheridan and Barrnett (1969) studied the reactivity of SC's from cultured lily microsporocytes after phosphotungstic acid (PTA) treatment. The lateral elements of pachytene complexes were highly reactive to PTA and stained strongly. Furthermore, the substance of this layer was extractable with dilute acid, and acetylation prior to staining inhibited the staining reaction. From their *in vitro* quantitative studies on the reactivity of isolated histones and deoxyribonucleohistones, the authors suggested that the SC lateral elements might well contain histones synthesized during early prophase.

In a recent communication on an Ascomycete of the genus *Neotiella*, Westergaard and von Wettstein (1970) report that DNase does not attack the SC's and that the central (sagittal) region together with parts of the banded lateral elements are highly sensitive to RNase. They conclude that the SC's consist of RNA and proteins.

In summary, these investigations indicate that most of the DNA is located in the peripheral chromatin of the bivalent, though some DNA may be located in the inner layer of SC lateral elements and the crossing space (Coleman and Moses, 1964). Histones are prominent in the structure of the lateral ribbons. However, since these researches were carried out in different species (plant, fungus, vertebrates), the results are only comparable in general terms. Cytotopochemistry has not yielded consistent results, possibly because preservation of material has generally been of poor quality, thus impairing a good analysis. Furthermore, most conclusions have been based on differences in electron density rather than on detailed scrutiny of structures. Still controversial issues are (1) the distribution of DNA in the lateral ribbons; (2) the presence of DNA in the pairing space; and (3) the existence of RNA among the layers of the complexes.

C. Development of Synaptonemal Complexes

The discovery of tripartite SC's in primary spermatocytes raised a problem regarding their origin and development. Moses' studies on the salamander *Plethodum cinereus* (1958) led him to the conclusion that the SC's arise from a joining of preexisting single units. On the other hand, Sotelo and Trujillo-Cenóz (1960) favored a different hypothesis: tripartite SC's would originate by the accumulation of material at both sides of a single elementary thread, so that each tripartite group would represent a single chromosome which would later pair with its homolog.

Moses' conclusions were based on a comparative study of prophase stages carried out with both the light and electron microscopes. In salamander spermatocytes, he found both single axial cores and SC's. Later, Moses and Coleman (1964) schematized the structure of SC's and represented each homolog as possessing a central or axial density which moves toward the axis of the bivalent when both homologs pair. Sotelo and Trujillo-Cenóz based their conception on images which suggested a transition from a single to a triple condition. The finding of five-partite groups in random sections of the species examined (Sotelo, 1962) contributed to make the hypotheses tenable. However, more recent observations on the same species have shown that five-partite groups are formed by a small segment of complex attached laterally to one of the ribbons of an autosomal SC (Wettstein and Sotelo, 1967, serial sections).

The presence of axial cores in leptotene chromosomes, as well as their participation in the formation of SC's, was studied by Franchi and Mandl (1962) in oocytes of young rats and by Moens (1968) in the sporocytes of *Lillium longiflorum*. Franchi and Mandl used the light microscope to establish the stages of prophase in relation to the animal's age (pre- and postbirth), then compared these stages with ultrastructure seen with the electron microscope. Moens correlated the stages of meiosis with the length of buds and then made a parallel study of specimens obtained from each bud using both light and electron microscopy.

Single leptotene densities or axial cores are very simple in structure. Examined at low magnification, they are similar in electron density to lateral elements seen at later stages, and like these they are enclosed in a poorly outlined field of microfibrils. They have been described as appearing in cross sections like small dots from which fibrils or filaments emerge radially (bristles). Their structure has also been studied in high resolution pictures (Sotelo and Wettstein, 1969), such as that shown in Fig. 12. The axial core seems to be formed by an undetermined number of filaments that run in a longitudinal direction for a short distance and then bend to join the field adjacent to the axial core, where

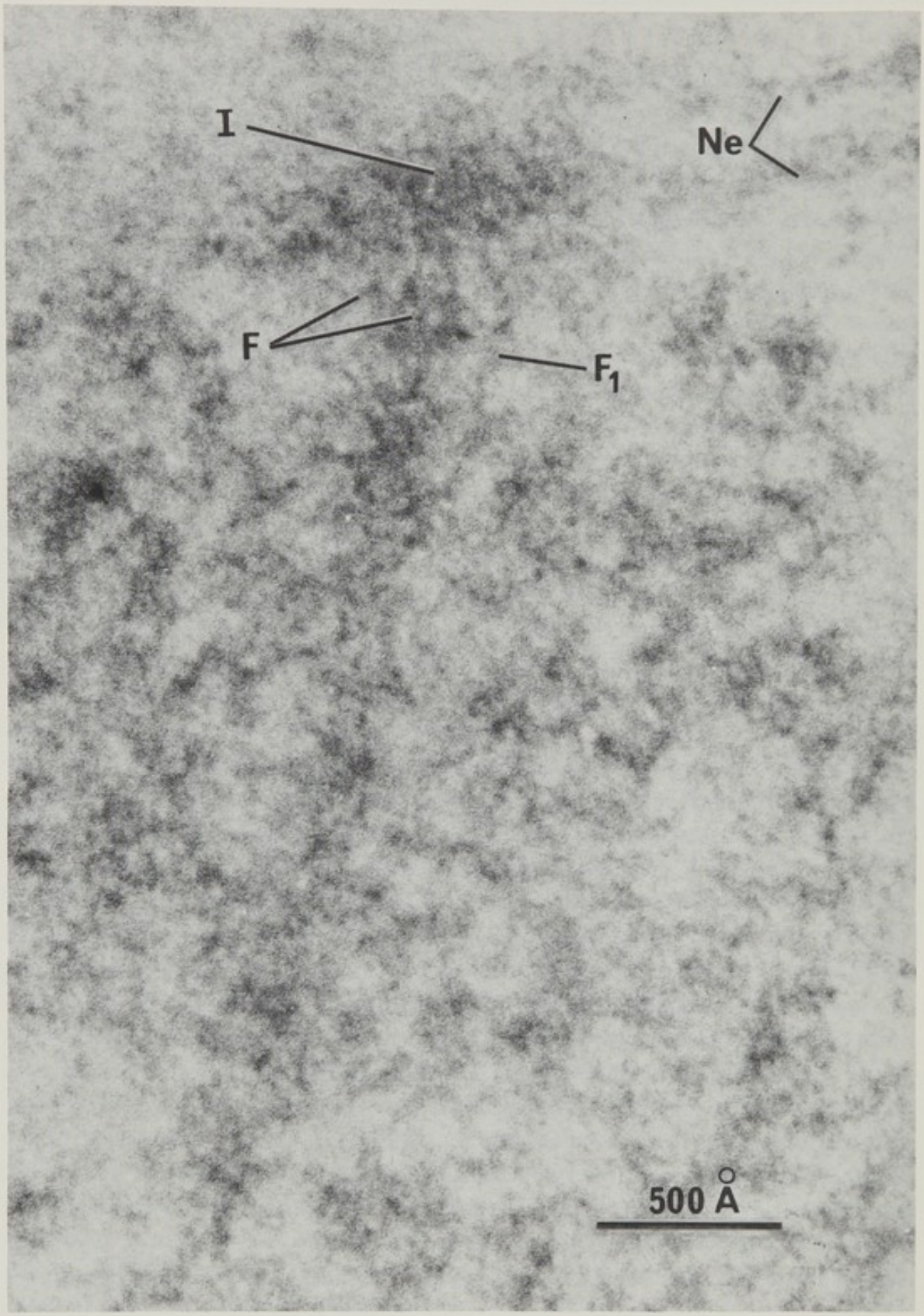


FIG. 12. This figure shows a leptotene axial core found in the cockroach *Blaptica dubia*. The oval density at the top of the figure corresponds to the insertion (I) on the inner membrane of the nuclear envelope (Ne); the latter was cut obliquely and therefore is not clearly seen in the micrograph. From this oval mass an undetermined number of filaments (F) arise. Some of them can be individually followed along a short trajectory (F_1), but the majority are soon lost to view. 460,000×.

they cannot be traced any longer. In some cases, the flow of filaments toward the adjacent chromatin occurs in an orderly way, and the axial core then appears to be transversely striated. Moens (1968) mentioned this orderly striation, and we have made a similar observation in spermatocytes of *Scaptericus borreli* Giglio Tos (unpublished). In *Locusta migratoria*, Moens (1969) found a highly regular distributive pattern (cross striations with a periodicity of about 100 Å).

The serial sectioning technique, applied to the study of single cores, has revealed that there is a stage—apparently preceding synapsis—in which the axial cores are incompletely formed. In *Periplaneta americana*, for instance, axial cores forming complete loops from one to another point of the nuclear membrane have been found, suggesting that they correspond to the total length of the leptotene threads (Sotelo, 1969); at the same time, these nuclei also contain short segments attached at one end to the nuclear membrane, but with the other end blended into the surrounding nuclear chromatin. A similar observation was made in a group of cytes from *Scaptericus* studied in a series of about 300 sections. These data suggest that the axial organization is initiated at separate points along the chromosome length.

It is not known whether in all species pairing begins between homologous chromosomes that have partially developed axial cores, or only after the axial cores have completely developed (i.e., are continuous from end to end). In *Locusta migratoria* Moens (1969) showed that synapsis begins near the nuclear membrane, at the centromeric and noncentromeric ends of the chromosome. Homologous chromosomes have their attachment points close together, so that some cores are co-aligned prior to synapsis. The most interesting finding reported by Moens (1969) is that the widest separation between cores for which cross-connecting filaments were observed is 3000 Å. He emphasized this fact, saying: "It therefore appears that cores are brought together to within 3000 Å by forces other than those involved in intimate pairing."

The close relationship between chromosomes of SC's and the nuclear membrane is the only fact which implies a predetermined position of chromosomes within the nucleus; this had already been noted by nineteenth century light microscopists and was observed by electron microscopists soon after the discovery of SC's. Whether they adopt the classic "bouquet" figure (Moens, 1969) or distribute randomly all over the nuclear contour, all the SC's of a chromosome complement are attached at both ends to the envelope. At the insertion point, the ends of the two SC lateral ribbons are cupped by the nuclear envelope layers, which appear darker than elsewhere. It is worth emphasizing that the contact is made precisely at the ribbon ends. It might be speculated

that this relationship is a consequence of the chromosome arrangement at the last gonial telophase, when endoplasmic reticulum cisternae close around the clump of chromosomes and established contact with them. Subsequent rounding up of the nucleus might not alter the acquired relationship except by moving the chromosome ends closer together or farther apart.

D. Experimental Work

The various problems associated with initiation of the meiotic process and subsequent stages cannot be resolved by simple observations of the cytological events as they occur in nature. Several investigators have resorted to different experimental approaches in order to discover the relationships between phenomena occurring at different phases of meiosis. The effect of elevated temperature on the production of chiasmata originally studied by Wilson (1959) prompted Nebel and Hackett (1961b) to experiment with this parameter on SC's. They determined the number of SC's in mouse spermatocytes as compared with single axial cores in random thin sections, and they concluded that high temperature blocks the formation of SC's. These results of Nebel and Hackett complemented observations made by Meyer (1964) in *Drosophila*, which indicated that SC's are absent in achiasmatic pairing (e.g., in male meiocytes of *Drosophila*) but present in chiasmatic pairing (e.g., in female meiocytes of the same species). Meyer's report also pointed out that *Drosophila* females homozygous for the mutant C(3)G^{+}, which is characterized by absence of pairing and chiasmata, lacked SC's; this mutant has since been studied extensively by Smith and King (1968, to be discussed).

A close relationship between DNA synthesis and formation of SC's was found by Roth and Ito (1967) who used another experimental approach. Previous experiments by Hotta *et al.* (1966) and Ito and Stern (1967) with cultured lily microsporocytes demonstrated that DNA synthesis is not limited to the premeiotic S period but continues during prophase; they found that this synthesis amounts to about 0.3% of the total DNA synthesis, and that if 2′-deoxyadenosine is used at the proper time to inhibit this activity, cytological abnormalities are produced. For example, inhibition of synthesis at leptonema leads to the result that the inhibited cells do not complete zygotene. Using the electron microscope, Roth and Ito (1967) showed that this is paralleled by the absence of synaptonemal complexes, and they demonstrated that part of the prophase DNA synthesis is necessary for the completion of pairing and associated formation of SC's. Two more discoveries of this experimental

work related to the ensuing phenomena of pairing are (1) DNA synthesis is required at late zygotene for the completion of disjunction, and (2) DNA synthesis is also required at pachynema for the separation of chromatids at anaphase II.

III. Synaptonemal-like Complexes

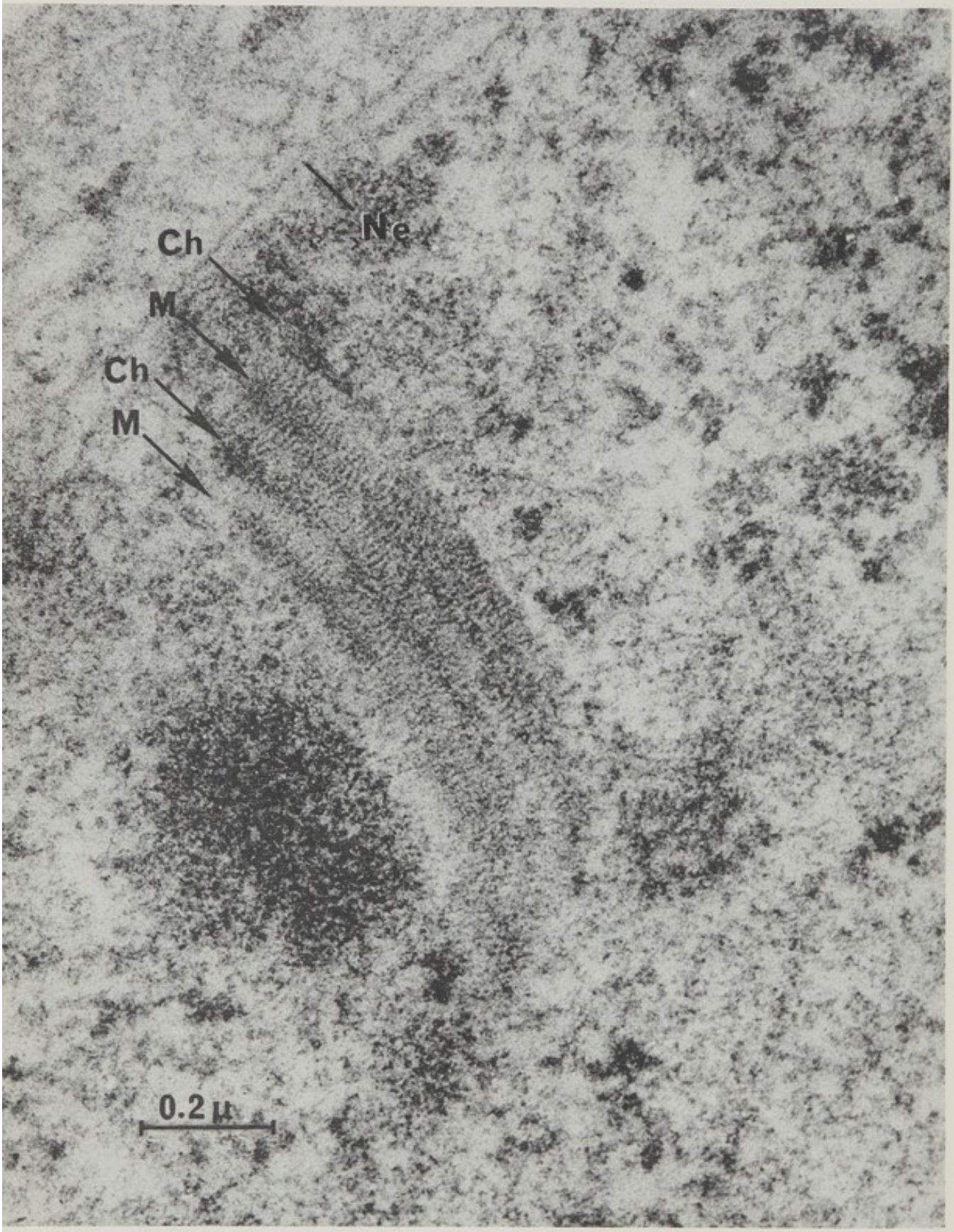
A. General

In spermatocytes, spermatids, and oocytes of Arthropods, it is frequent to find structures which, though morphologically like SC's, are not associated with any orthodox pairing of autosomes. These structures are supernumerary in relation to the SC's which correspond to the haploid number in the species concerned, and they sometimes associate with other nuclear components like the nucleolus, the sex chromosome, or some autosomal complexes. These structures may change during the course of prophase (or spermiogenesis) from the primary ribbonlike state to multiply stacked lamellae or crystalloid bodies. The characteristics mentioned above are implied in the names they have received: synaptonemal-like complexes (SLC), extracomplexes or polycomplexes.

Sotelo and Trujillo-Cenóz reported the first examples in two insect species: *Gryllus argentinus* (1960, 1961) and *Blaptica dubia* (1960). Similar structures were later found in European and North American Gryllidae species (Schin, 1965a,b; Guénin, 1965; Moses, 1969), in the Homoptera *Philaenus spumarius* (Folliot and Maillet, 1966) in the Opilion *Acanthopachyllus aculeatus* (Wettstein and Sotelo, 1965b) and in the mosquito *Aedes egipti* (Roth, 1966). It is extremely difficult to give a unitary view of the different types occurring in nature, as well as those found by Sotelo and Wettstein (1969) under experimental conditions (Fig. 13). Therefore, we will first describe their common features and then the variations of form.

B. Common Features

SLC's are present only in gametocytes or in cells parentally related to them. In the literature, there is no case reported of SLC's occurring in somatic cells except for the nurse cells of the Diptera species studied by Roth (1966); however, Roth noted in this regard that nurse cells have the same origin as the oocytes they surround. SLC's repeat the structural pattern of the autosomal SC's. This fact is particularly obvious in cases where the medial ribbon shows a precise array of components.


Ne
Ch
M
Ch
M
0.2 μ

C. Variations of Form

In *Gryllus argentinus* spermatocytes, as well as in those of other Gryllidae examined (see literature cited above), various types of SLC have been found: (1) heterologous complexes formed by the chromatin material of the X-chromosome on one side and by nucleolar material on the other; (2) complexes constituted by medial ribbons lying inside nucleolar channels; (3) tubular complexes, formed as a cylindrical layer built on the pattern of a medial ribbon and coated by nucleolar material (Fig. 14); and (4) short segments of complexes adhering to a lateral element of one of the autosomal SC's. The first type is illustrated in Fig. 2A; it has two different segments: the shorter one is flanked on both sides by nucleolus and sex chromatin, whereas the longer one lies free in the nucleus and ends at a random place on the nuclear membrane. This part of the SLC cannot be differentiated from autosomal SC's unless traced in serial sections. During pachytene the complexes associated with the nucleolus–sex chromatin evolve into two heterogeneous masses, which can be found among the chromosomes until the end of diplotene.

In spermatids of Gryllidae, there have been found (1) stacks of SLC's connected to a chromatin-like mass, (2) stacks of SLC's—mostly lamellar in shape—connected to a round body, and (3) stacks of SLC's free in the nucleus. Sotelo and Wettstein (1964b) suggested that the chromatin material and the round body are the X-chromosome and the nucleolus, respectively, and a similar proposal was made by Schin (1965b). However, Wolstenholme and Meyer (1966) demonstrated that the chromatin material was present in all spermatids (the X should be present in only half of the spermatids) and that the round body contains DNA.

In *Acanthopachyllus aculeatus* (Opilion), SLC's associate at early pachynema to form a stack of parallel units, which during the course of pachynema changes into a crystalloid body showing a hexagonal pattern in cross sections. In spermatids of the cockroach *Blaptica dubia* (Fig. 15), groups containing two to four short complexes are found immersed in dense chromatin blocks. In spermatocytes of the homopteran *Philaenus spumarius*, up to 14 alternating clear and dark bands were counted at prophase by Folliot and Maillet (1966). However, the authors

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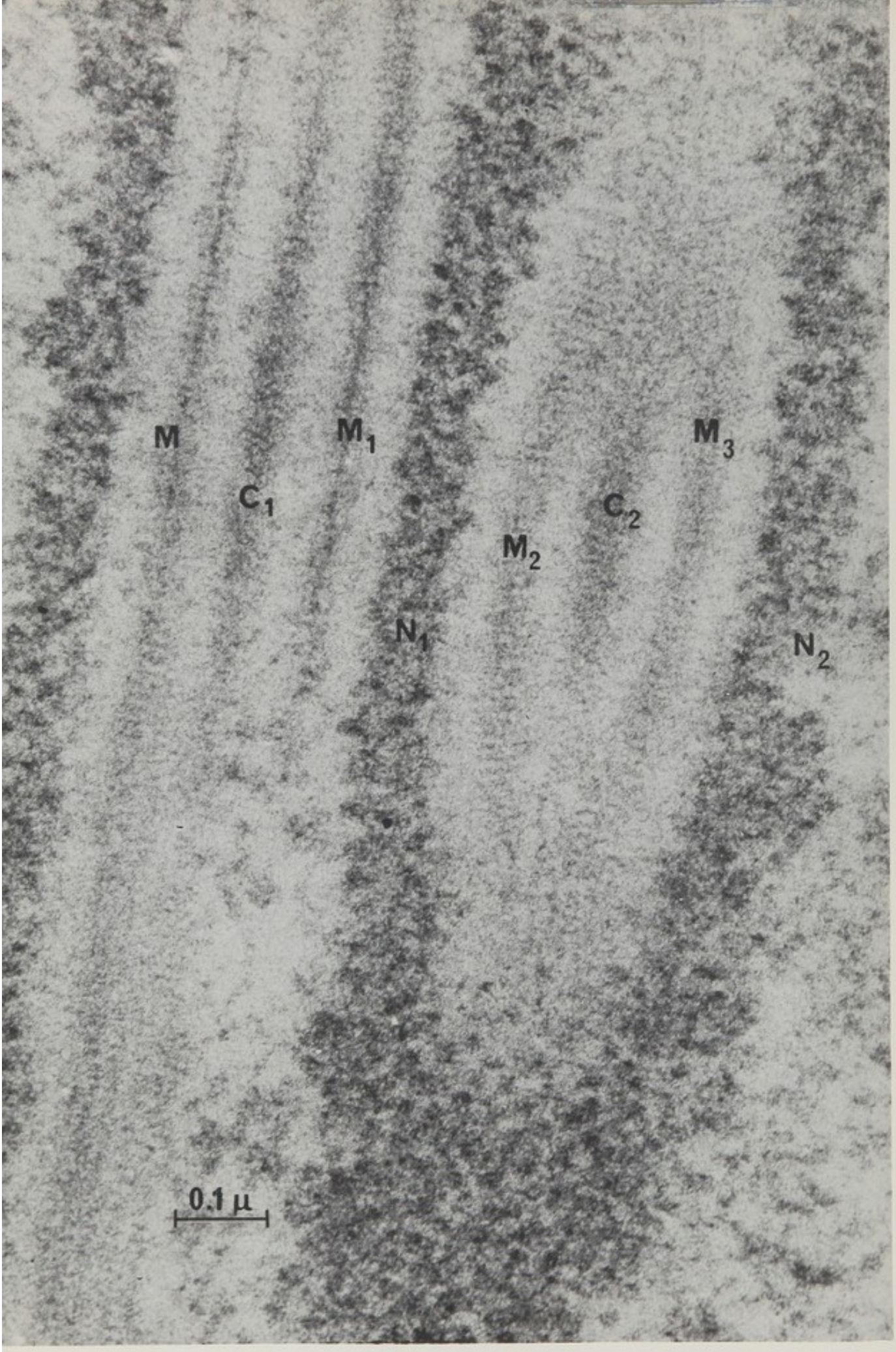
Fig. 13. Irradiated specimens of *Gryllus argentinus* show structures like the one seen in this picture. They are composed of two or more layers of medial-like material (M) which has an organization like that illustrated in the normal SC of Figs. 10 and 11. Multilayered complexes or cylindrical forms (like the one in Fig. 14) are considerably more frequent after irradiation. (Ne) nuclear envelope; (Ch) chromatin-like material interposed between the medial-like layers. 100,000×.


M
M1
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N2
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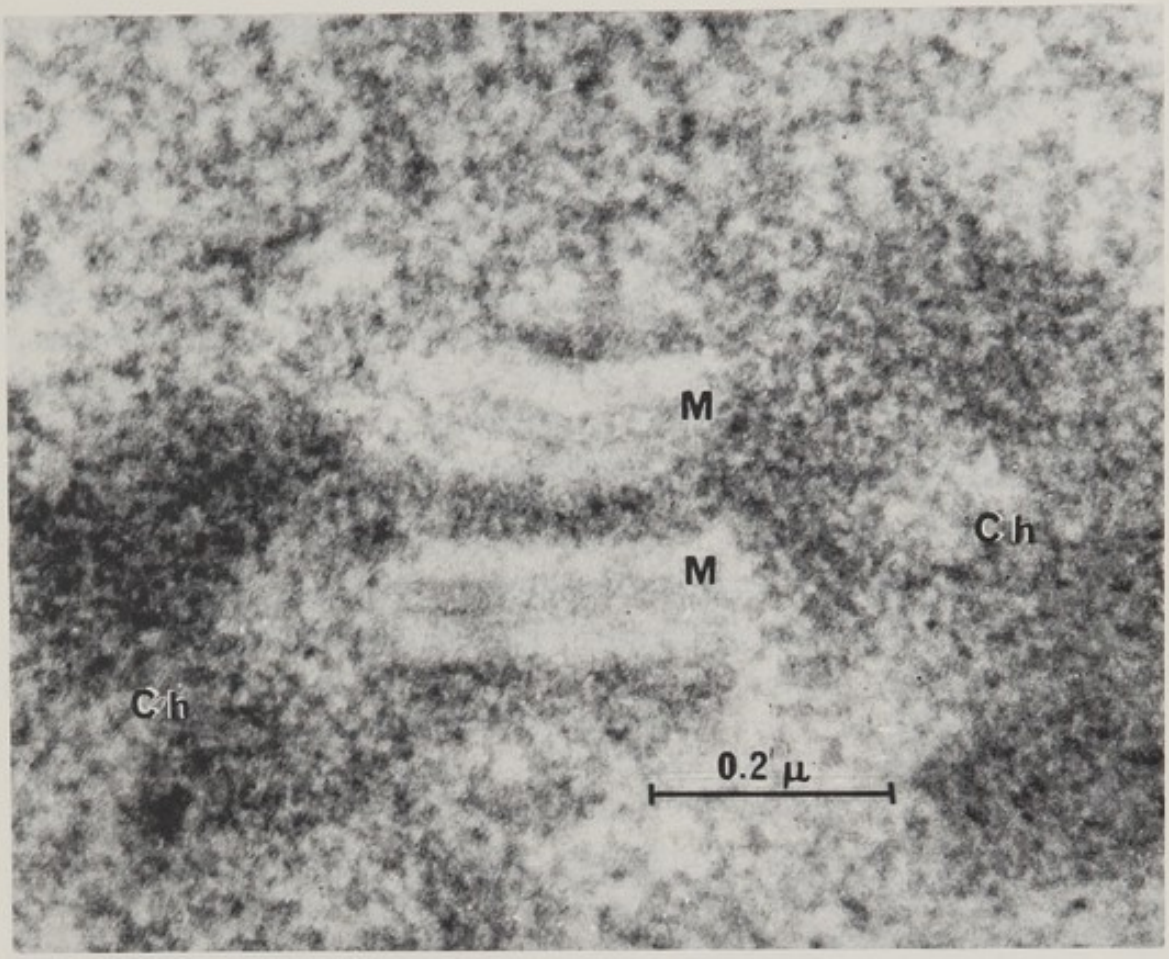

FIG. 15. In spermatids of the cockroach *Blaptica dubia*, groups formed of 2 to 4 short synaptonemal-like complexes (two in this case) are frequently found in the middle of a dense chromatin-like mass (Ch). Note that the medial ribbon (M) has the two parasagittal planes characteristic of SC's in cockroaches 120,000×.

did not provide detailed information about the time of appearance of these structures. In mosquito oocytes, Roth (1966) found large aggregations of SC's. The fact that these structures, which he called polycomplexes, appear at the beginning of disjunction and that at least some of the most peripheral elements of the polycomplex are coated with chromatin material led him to the conclusion that the aggregation takes place after shedding of chromatin from the SC or vice versa. From the observations made by Roth it seems that the polycomplexes increase in size and number of units as prophase advances.

FIG. 14. This electron micrograph illustrates only a small part of the tubular component mentioned in the text. Furthermore, the tubular component is double in this particular association. The structural pattern of the elements marked (M), (M_1), (M_2), and (M_3) is similar to that found in the pairing complexes of the autosomes in this species. (N_1), (N_2) nucleolar material; (C_1), (C_2) chromosomal material. (From Sotelo and Wettstein, 1966b.) 180,000×.

From the above cited descriptions, the differential characteristics of SLC's reported up to now may be summarized as follows: (1) They differ in time of occurrence during gametogenesis, including spermatocytes at early prophase, spermatocytes at late prophase, and spermatids; (2) they differ in type of association, including association with a nucleolus and/or sex chromatin, association with autosomal complexes in spermatocytes, or self-assembly of multiple segments in spermatocytes and spermatids.

IV. General Discussion

SC's present the following main problems: (1) their origin and relationship to chromosomes; (2) their composition and structural organization; (3) their function.

A. Origin

Some of the available information suggests that SC's originate by the joining in pairs of single cores or densities which appear in the axes of leptotene chromosomes (Baker and Franchi, 1967a; Moens, 1968, 1969). Approximation of these axial cores implies changes in the loose material, or chromatin surrounding them. It has been suggested that part of this material becomes incorporated in the structure of the crossing filaments and medial ribbon (cf. Moses, 1964, 1968; Moens, 1968).

Although most facts point to the axial cores as the predecessors of the SC lateral elements, it is not known whether they originate by the linear reordering of the pre-existing DNA–protein complex or whether they are synthesized "de novo." Certainly the appearance of axial cores and the presynaptic linear organization is the first landmark differentiating the diploid and haploid cycles; no array of this kind has ever been observed in the prophase chromosomes of somatic cells. It seems reasonable to postulate that their appearance is a necessary prelude for site by site pairing, and it is also tempting to associate the formation of new structures at leptonema with the small amount of DNA synthesis discovered by Hotta *et al.* (1966) during the same stage. Nevertheless, no data have been reported as to whether inhibition of DNA synthesis blocks the formation of axial cores as it does the formation of SC's. Furthermore, little is known about the distribution of DNA among the structural components of the complexes (see Section II,B); that proteins are synthesized concomitantly with the joining of axial cores has been

demonstrated by Hotta *et al.* (1968), who found that the progress of meiosis through zygotene is arrested if protein synthesis is inhibited by cycloheximide (nonhistone proteins). In this regard Stern and Hotta (1969) have commented that ". . . it cannot be answered whether the complexes are formed from proteins synthesized at zygotene or whether its formation depends upon protein synthesis for the production of adequate enzymes or accessory structural elements." That this protein synthesis is gene dependent is indicated by the fact that the structural characteristics of the medial lattices in a given species tends to be constant through successive generations. However, the extent to which protein participates in the production of ordered structures and the extent to which DNA does is not known.

B. Composition and Structural Organization

It has already been noted in this chapter that the data on chemical composition of SC's are ambiguous. The possible occurrence of DNA in the bivalent layers has been studied by different researchers (see Section II,B), but their results do not agree convincingly. For example, Nebel and Coulon (1962b) concluded from DNase-digested samples that lateral elements are not attacked by this enzyme, while Coleman and Moses' (1964) cytochemical tests indicated that their inner layers contain DNA. Although Westergaard and von Wettstein's findings supported Nebel and Coulon's results, they also reported that the complexes are attacked by RNase which Nebel and Coulon found to be inactive. Improvements in the methods for "in situ" detection of both nucleic acids seem necessary.

The experimental work described in Section II,D has shown that DNA and protein are synthesized concomitantly with the formation of the pairing structures. Inasmuch as most studies on chromosome fibrils of different origin have demonstrated that DNA and protein are closely associated (see Ris, 1967, 1969), it is likely that at least the peripheral fibrils of the bivalent contain DNA; furthermore, it is in the zone occupied by these fibrils that cytochemical tests consistently show that DNA is present. The dissociation of components observed in the transition zone between peripheral chromatin and lateral ribbons might mean that there is a dissociation of DNA from protein. However, the only data available in regard to this point indicate that the fibrils break up at the outer limit of the lateral elements to give rise to a different distribution of elementary filaments (compact or ordered lateral elements, see Section II,A,2). Therefore, if DNA and protein are also asso-

ciated in the lateral ribbon and/or the pairing space, this association must be entirely different from that existing in the peripheral chromatin.

The problem of whether DNA occurs in the three main layers of the SC brings up another question, this one involving both the chemical composition and structure. As mentioned in Section II,A,2 and 3, several authors have recognized two subunits in each lateral element, which they identified as sister chromatids (Nebel and Coulon, 1962a; Woollam and Ford, 1964). This division of lateral elements into chromatids, as well as some other interpretations of SC structure such as the one attempted by Moens (1968, see below), imply the presence of DNA in the lateral elements and in the pairing space as well.

A last topic for consideration in this respect is the subject of synaptonemal-like complexes, some of which are considered to be expended SC's. Moses' investigations (1969) using indium trichloride to stain DNA in spermatocytes and spermatids of Acheta (Gryllidae) led him to report indium-positive material in the medial regions of both SC's and SLC's; however, he states that the evidence cannot be considered unequivocal. If it turns out that DNA is present in both cases, we would have to conclude that not all the DNA synthesized at the pre- and meiotic prophase is used by the cells at later stages, and that this DNA is synthesized only as part of the structure of the pairing mechanisms.

The existence of SLC's which outnumber the haploid number of the species (*Gryllus argentinus* spermatocytes; Wettstein and Sotelo, 1967) implies that there may be complexes not specifically related to bivalents [in fact, axial cores not related or partially related to prospective pairing have also been found; e.g., in the sex chromosome of *Gryllus* oocytes (Sotelo and Wettstein 1964b) and sex chromosomes of mouse spermatocytes (Solari, 1969)]. To explain some of the situations encountered, some authors have postulated that there may be homology between different loci of the same chromosome (Schin, 1965b); however, this hypothesis is difficult to apply to small segments attached laterally to an autosomal SC, or to the long extracomplex which arises in the sex chromosome and ends far away from it.

The following points stand out: (1) SC's and SLC's are built according to the same structural pattern; (2) this structural pattern is species constant (Sotelo and Wettstein, 1966b; Sotelo, 1969); (3) in some species SC's are thought to be shed from the chromosomes (Roth, 1966); (4) SC's or parts of them may remain undestroyed until anaphase I (Gassner, 1969). Points 1 and 2 would indicate that the structural pattern is codified in the genome. Points 3 and 4 bring us back to points made in previous paragraphs about the discarding of part of the DNA.

Considering that the ultrastructure of the pairing mechanism is a species characteristic, we can postulate that synthesis of the materials required for its construction should be under control of genic factors which are active only at the preleptotene or leptotene period. If these factors are not repressed at the appropriate time and loci, they may give rise to complexes not properly related with synapsis, which may then involve other nuclear components as well. Shedding of SC's and their mutual aggregation may mean a partial failure of the enzymatic mechanism normally acting in other species at disjunction. Such shedding does not seem to occur frequently in nature, and even less frequent is the permanent presence of SC segments until anaphase and telophase (e.g. the situation reported by Gassner and illustrated in Fig. 16).

When disturbances were induced by ionizing radiation in the enzymatic mechanisms regulating the equilibrium of structures involved in synapsis and related phenomena (Sotelo and Wettstein, 1969), there was a considerable increase in SLC's of varied forms (cylindrical complexes and multiple-layered complexes). In these experiments, hyperproduction of structures mostly involved medial-like material which presented itself as groups of parallel layers among which little amorphous material was interposed (Fig. 13). Cylindrical structures, which in normal *Gryllus* spermatocytes are found only associated with the nucleolus, were found elsewhere in irradiated nuclei, some of them evidently connected to autosomal SC's. Moens' electron micrographs of *Locusta migratoria* spermatocytes (1969) also indicate that double medials can sometimes form at junction time; this is a spontaneous phenomenon of doubleness similar in many respects to the parallel double or triple medial components that appear in *Gryllus* nuclei after irradiation.

In conclusion, although little experimental work has been done on SLC's, it is probable that at least some of these structures form due to a failure in the mechanisms governing synthesis; in some species, this failure has become a hereditary characteristic.

C. Function

The fact that SC's appear synchronously with the period in which nonsister interchromatid exchanges take place suggests that they facilitate this important goal of meiosis. However, the true sites where genetic interchanges occur are unknown. It seems certain that close approximation between DNA molecules is essential for effective pairing so that

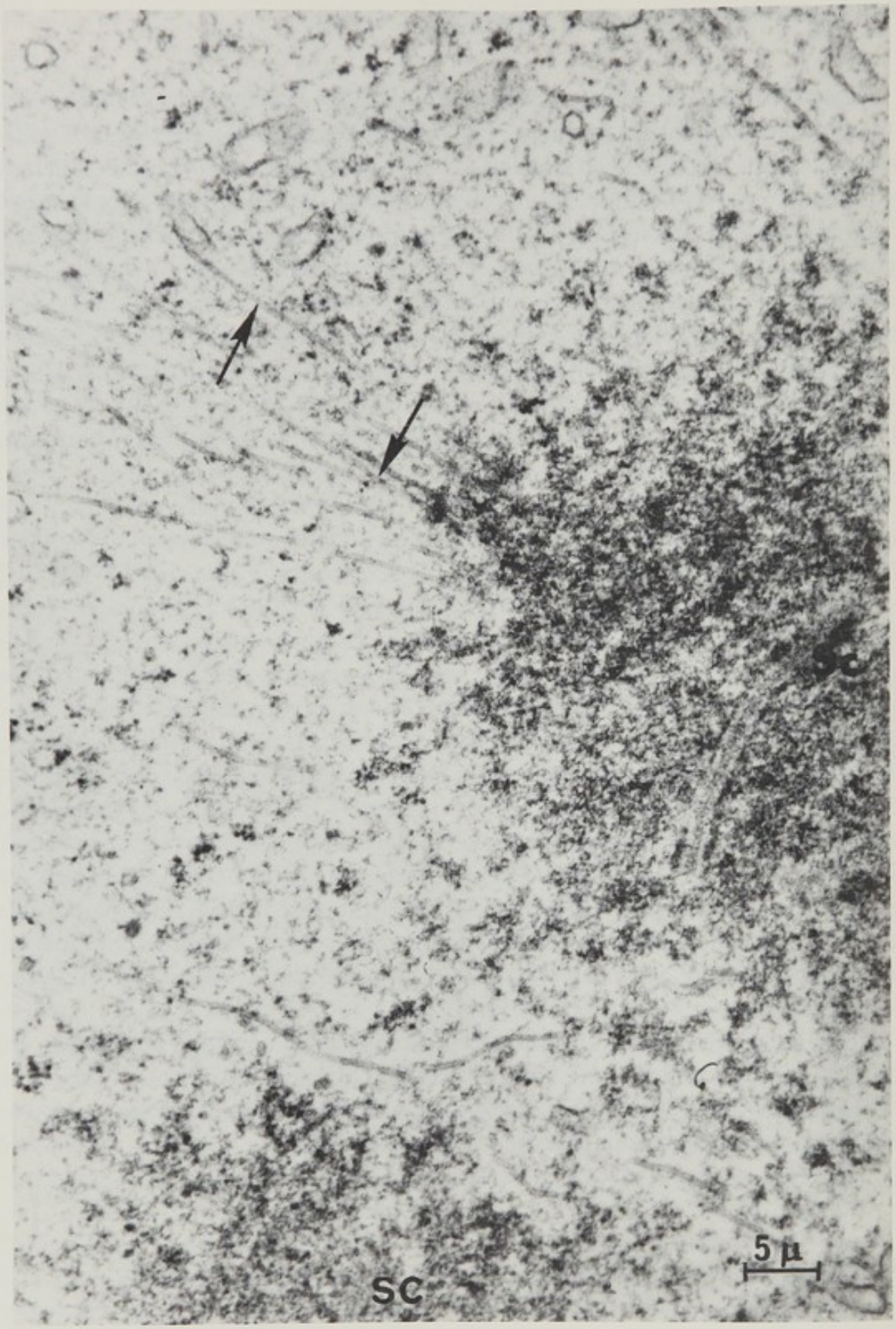

FIG. 16. In the achiasmatic spermatocytes of *Bolbe nigra* (a Mantid), Gassner (1969) found synaptonemal complexes not only at prophase but also at later stages such as metaphase and anaphase I. This figure shows a synaptonemal complex (SC) at early metaphase I of meiosis. Spindle fibers are clearly seen in the upper left part of the figure. (Courtesy of the author.) 20,000×.

exchange between homologous DNA segments may occur. However, uncertainty about the localization of DNA makes it difficult to decide which of the three differently organized components forming the bivalent (peripheral field of chromatin fibrils, lateral elements, or medial ribbon) can be involved in interchange. So far, two sites are known in which intermixture of components from both sides take place: (1) the pairing space; and (2) the two spaces outside the SC where the chromatin fibrils of the two homologs interlace (see Fig. 1). Involvement of the pairing space in cross-over events was suggested by Meyer (1964), who estimated that the number of cross-connecting filaments is about 1000–2000 per bivalent, approximately the total number of genes per large autosome of *Drosophila melanogaster*. According to Meyer, this suggests that there may be one cross-connecting filament per allelic pair. This inference would be significant under the following conditions: (a) serial sections about 500 Å thick to estimate the length of the bivalent; (b) determination, for the species concerned, of the elementary units of crossing (single filaments or filaments associated in fibrils); and (c) counting of bridging units in several cross sections of the same bivalent at magnifications above 200,000×. In addition to this, of course, it still has to be demonstrated whether or not the crossing units contain DNA.

That SC's are involved in chiasmata and crossing over has been inferred from their absence in achiasmate meiosis (Meyer, 1964). However, after the early report by Meyer, other researchers found examples both confirming and contradicting this relationship. For instance, Smith and King (1968) studied the oocyte nuclei of homozygous C(3)G^{+} females of *Drosophila*, as described in Meyer's report, and found that the C(3)G^{+} gene is required for the synthesis of the SC subunits; abolition of crossing over by the suppressor gene is paralleled by the abolition of SC's. On the other hand, Gassner (1967, 1969) found two achiasmatic species, *Panorpa nuptialis* and *Bolbe nigra* which possess SC's; Menzel and Price (1966) also discovered in nonhomologous pairing (hybrids between *Lycopersicum esculentus* and *Solanum lycopersicoides*) no evidence of crossing over or chiasmata, but normal SC's. These cases were interpreted by Moses (1968) as follows: "If a generalization is to be drawn from such observations, it can only be that the synaptinemal complex (and effective synapsis) must be present for chiasmata to be formed, but that chiasmata do not necessarily follow from synaptinemal complex formation."

As a complementary comment to this point it is interesting to recall that (1) chiasmata are recognized as physically visible phenomena only at the end of pachytene or diplotene, and (2) in serial studies of

pachytene chromosomes, it has not been possible to find indications of how future chiasmata will form (cf. Moens, 1969).

As reported by Stern and Hotta (1969), low concentrations of cycloheximide partially and selectively inhibit protein synthesis and simultaneously arrest meiotic development. Suppression of the inhibitor allows the cells to continue meiosis. If the inhibitor is applied at late zygotene or early pachytene, the consequence is that the cells continue to develop but chiasmata are not formed. This type of experiment fixes the time of action of the mechanism responsible for chiasmata formation between late zygotene and mid-pachytene, although at this time chiasmata are not yet visible in the bivalents (see above).

A general interpretation of SC's requires not only consideration of the circumstances associated with their formation and presence throughout meiotic prophase, but also integration of the observed morphology with proposed models of chromosome structure. Baker and Franchi (1967b) on the basis of their electron microscope observations on diplotene chromosomes of primordial human oocytes (Fig. 17), proposed a diagram (Fig. 18) to explain the structure of chromosomes at meiosis; this may be compared with the one proposed by Nebel and Coulon (1962a,b) or the one by Moens (Fig. 19). In all three cases, loops are drawn as extensions of dense longitudinal strands running along the axis of each homolog. These dense strands have been found in chromosomes from leptotene through diplotene (at synaptene and pachytene as parts of the synaptonemal complexes), and may well form the backbones of the chromosomes. All the diagrams except Moen's have been used only to represent morphology and have not been applied to develop any special hypothesis of the molecular structure of meiotic chromosomes (i.e., axial cores and synaptonemal complexes).

An integration of electron microscope observations with a hypothesis explaining molecular structure in relation to genetic function was attempted by Moens (1968), who adopted the cycloid model proposed by Whitehouse (1967). Moens' interpretation is based on the assumption that (1) the chemical and structural characteristics of the molecules composing the SC lateral elements are similar, if not identical, with those of the axial cores, and (2) the 100 Å DNA-protein chromosome fibers form loops which originate and terminate at the lateral elements, and which do not participate directly in genetic exchange. Whitehouse's model will be discussed only to the extent necessary to introduce Moen's elaborate diagram (Fig. 19). The model was devised to bring into conformity the fact that each gene may be represented many times in a chromosome with the evidence, particularly striking for lampbrush chromosomes, that each chromosome is single-stranded.

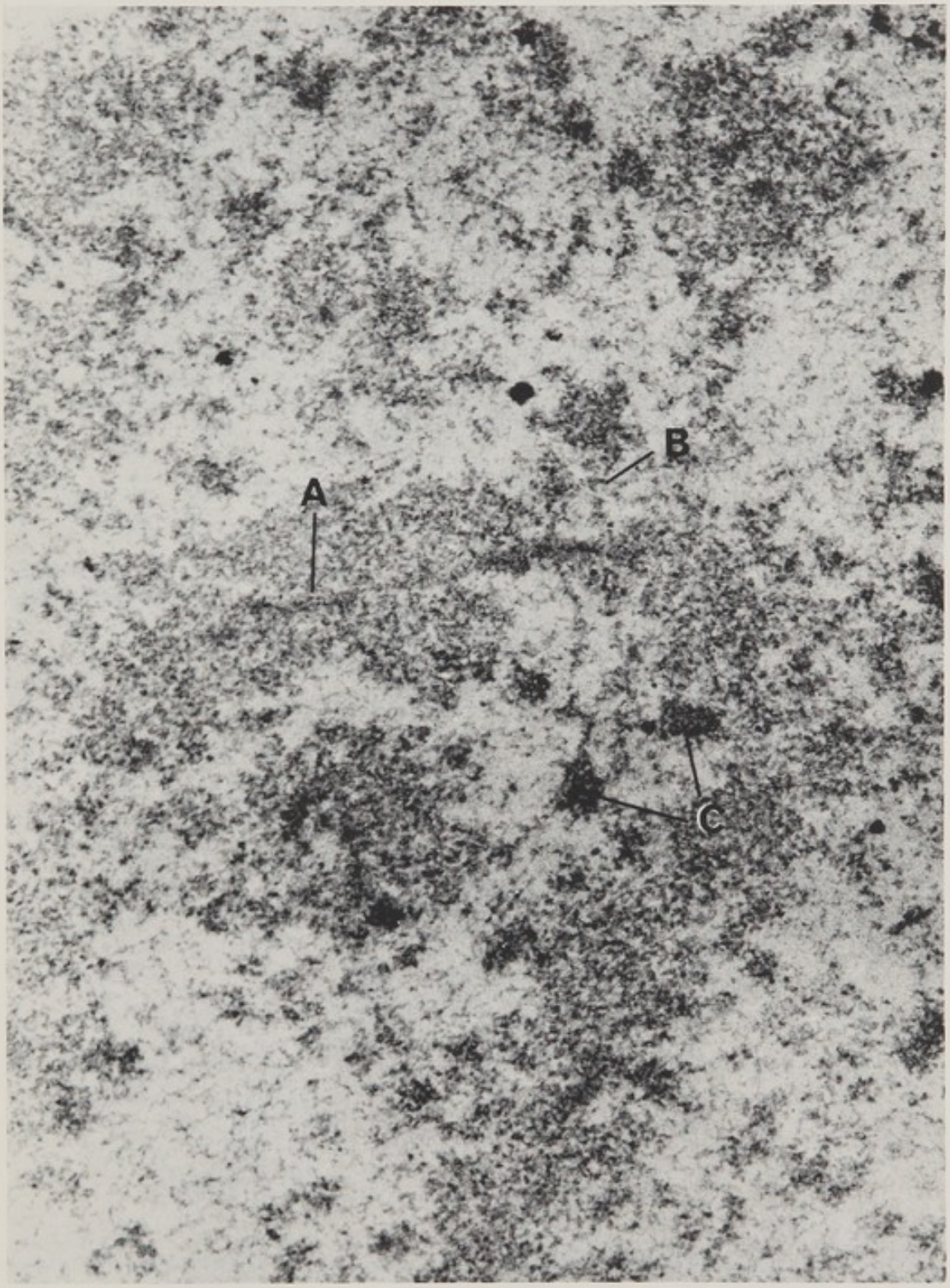

FIG. 17. Electron micrograph of a part of a human oocyte chromosome. The axis (A) shows two major strands, from which emerge fibrillar projections and loops (B) with their associated granular clusters (C). Human primary oocyte at diplotene, 39,000×. (From Baker and Franchi, 1967b.)

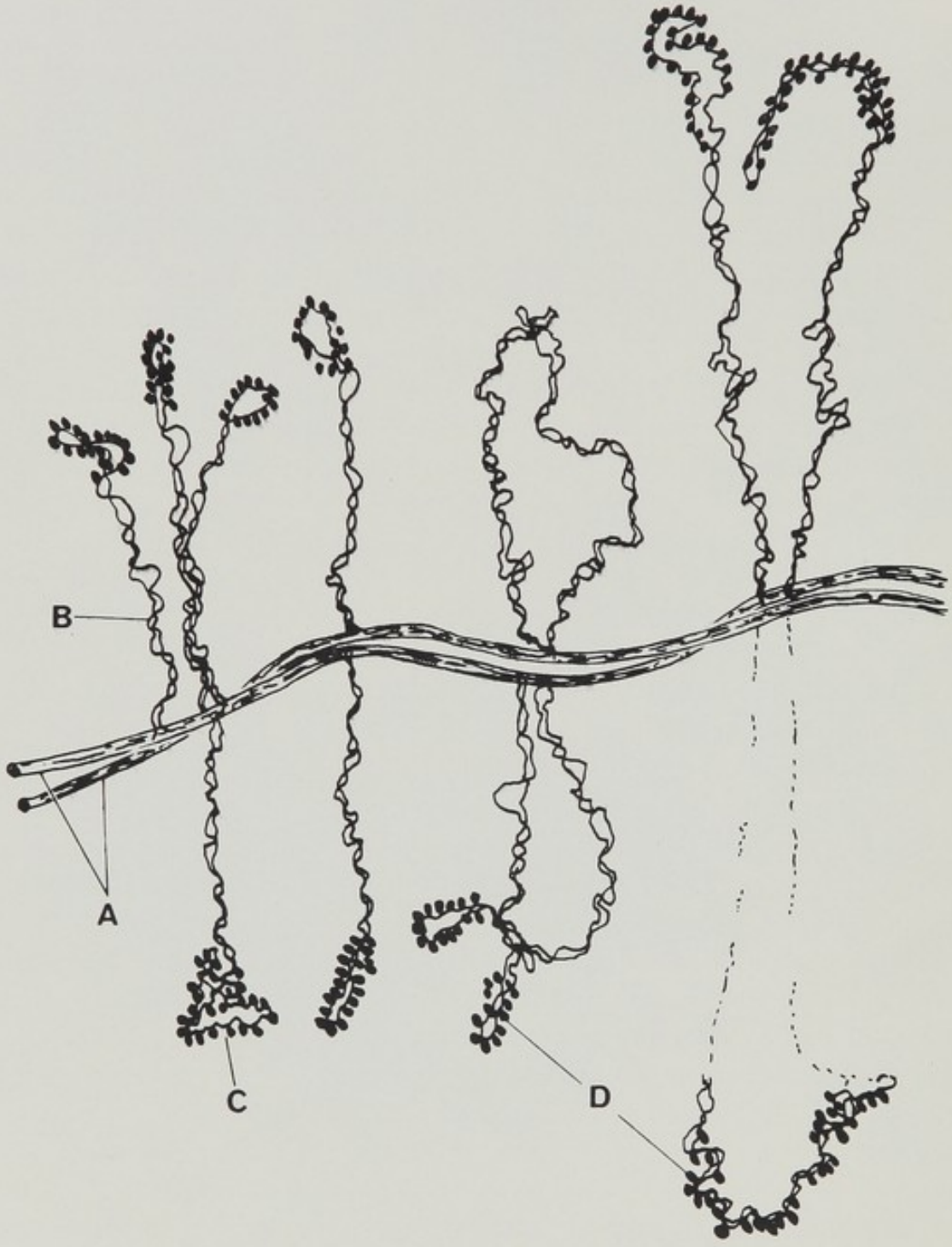

FIG. 18. Diagrammatic interpretation of the structure of a human oocyte chromosome at diplotene, based on electron microscopy. The homolog is composed of: (A) Axial core of the chromosome; there may be more than the two strands shown here. (B) Coiled fibrils emerging from the axis and forming lateral projections; numbers of these crowded together collectively form the fibrillar sheath of the chromosome. (C) Clusters of granules at the ends of the projections; the diagram indicates a possible mode of attachment to the fibrils. (D) Loops formed by the reflection of projections back to the axis. These features are illustrated in the electron micrograph shown in Fig. 17. (From Baker and Franchi, 1967b.)

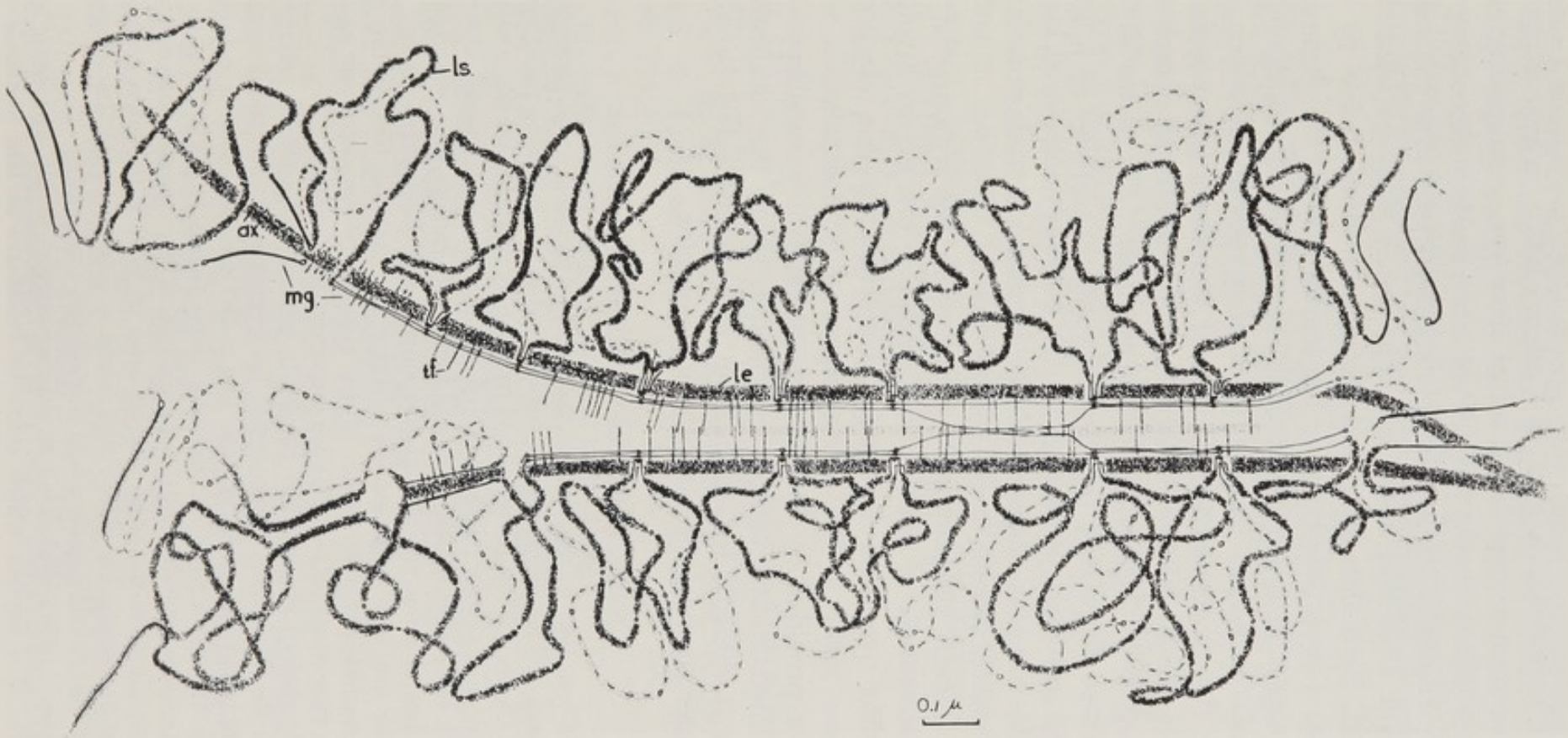

FIG. 19. A drawing which illustrates the axial cores (ax) at leptotene, the pairing of two axial cores during zygotene, the completed synaptonemal complex during pachytene, and the disaggregation of the complex at the end of pachytene. The hypothetical arrangement of the chromatin around the complex is meant to convey the idea that the axial cores and later the synaptonemal complex function to organize the recombinationally active genes into the proper arrangement for genetic recombination to take place. In this model, Callan's master genes (mg) are shown forming a linear sequence along the core while the slave copies become loops fixed to the outside of the core. (ls), lateral strands; (tf), transversal filaments; (le), lateral elements. (From Moens, 1968.)

A comparable hypothesis is that of Callan (1967), which proposes the existence of a master gene and a set of slave genes composing each chromomere, the slaves being copies of the master. According to Whitehouse, the copies of each gene are removed from the chromatid at cross over, and after this event the copies may then be reinserted in the chromatid. Both detaching and reinsertion would occur by the same mechanism: a cross over between the master gene in the chromatid and one of its copies in the corresponding ring. Whitehouse's diagrammatic scheme of this model has the form of a cycloid, of which the loops represent the chromomeres. The most significant characteristics of the model are the continuity of DNA and the ability to detach the loops by crossing over. According to Moens' article: "In terms of the cycloid model those loops may represent the isolated strands of copies. Directly involved in meiotic exchange are DNA strands associated longitudinally with the complex. There would be four such strands of isolated master genes or master strands, in the cycloid model."

Even though Moens' diagram may oversimplify the structure of an SC, it has the advantage of encompassing most of the events involved in pairing, crossing over, and disjunction. Unfortunately, his interpretation of the linear array in the pairing space does not coincide with most of the data provided by high resolution electron microscopy (cf. Sotelo, 1969). Furthermore, lack of information about the localization of DNA makes it difficult to establish an exact relationship between the inner and outer layers of the synaptonemal complex.

NOTE ADDED IN PROOF

Since this article was sent to the printers, information pertinent to the subject of the article has appeared. This information is summarized in the following paragraphs:

1. Comings and Okada (1970a,b) published two papers reporting on the structure of the SC as seen in electron micrographs of whole mounts of spread chromosomes. The authors were able to see in mouse and quail enough long stretches of SCs in which the lateral arms, the medial component, and the bridges uniting the latter to the former are very apparent. In addition, the authors treated the SCs with DNase and concluded that no action of this enzyme can be perceived on this material. Therefore they conclude that SCs are proteinaceous in nature.

2. Moens (1970) studied chromosome pairing in natural and artificial polyploids. Switching between the 4 partners (represented by switching of the lateral arms of SCs) of the autotetraploid *Lilium longiflorum* ($4n = 48$) and abnormal or deformed lateral elements in the allotriploid *L. tigrinum* were found in serial sections of microsporocytes. According to the author inequalities in the genetic material will be expressed as inequalities in the cores (lateral arms of SCs) if the latter represent the genetic material in meiosis. The deformities found in heteromorphic "homologous" chromosomes of the allotriploid species would support this assumption.

3. Hotta and Stern (1970) discovered in microsporocytes of lily at meiotic prophase the existence of a protein which starts to synthesize at late leptotene and

reaches a maximum at mid-pachytene. This protein binds strongly to single-stranded DNA or to double-stranded DNA having gaps or nicks.

4. Howell and Stern (1970) report that at zygotene-pachytene in lily microsporocytes, activity of a polynucleotide ligase has been found to appear at the period of pairing of homologous chromosomes; they propose that this activity may be related to the breakage and reunion mechanism responsible for genetic recombination.

REFERENCES

Baker, T. G., and Franchi, L. L. (1967a). *J. Cell Sci.* **2**: 213.

Baker, T. G., and Franchi, L. L. (1967b). *Chromosoma* **22**: 358.

Callan, H. G. (1967). *J. Cell Sci.* **2**, 1.

Coleman, J. R., and Moses, M. J. (1964). *J. Cell Biol.* **23**: 63.

Comings, D. E., and Okada, T. A. (1970a). *Nature* **227**: 451.

Comings, D. E., and Okada, T. A. (1970b). *Chromosoma* **30**: 269.

DuPraw, E. J., and Bahr, G. F. (1969). *Acta Cytol.* **13**: 188.

Fawcett, D. W. (1956). *J. Biophys. Biochem. Cytol.* **2**: 403.

Fawcett, D. W. (1966). "An Atlas of Fine Structure. The Cell, its Organelles and Inclusions." Saunders, Philadelphia, Pennsylvania.

Folliot, R., and Maillet, P. L. (1966). *C. R. Acad. Sci. Ser. D* **262**: 394.

Franchi, L., and Mandl, A. M. (1962). *Proc. Roy. Soc.* **B157**: 99.

Gall, J. G. (1956). *Brookhaven Symp. Biol.* **8**: 17.

Gall, J. G. (1963). *Nature* **198**: 36.

Gassner, G. (1967). *J. Cell Biol.* **35**: 166A.

Gassner, G. (1969). *Chromosoma* **26**: 22.

Guénin, H. A. (1965). *J. Microscop.* **4**: 749.

Guénin, H. A., and Gautier, A. (1960). *Rev. Suisse Zool.* **67**: 210.

Hotta, Y., and Stern, H. (1970). *J. Cell Biol.* **47**: 91a.

Hotta, Y., Ito, M., and Stern, H. (1966). *Proc. Nat. Acad. Sci. U.S.* **56**: 1184.

Hotta, Y., Parchman, L. G., and Stern, H. (1968). *Proc. Nat. Acad. Sci. U.S.* **60**: 575.

Howell, S., and Stern, H. (1970). *J. Cell Biol.* **47**: 92a.

Ito, M., and Stern, H. (1967). *Develop. Biol.* **16**: 34.

Menzel, M. Y., and Price, J. M. (1966). *Am. J. Botany* **53**: 1079.

Meyer, G. F. (1964). A Possible Correlation between the Submicroscopic Structure of Meiotic Chromosomes and Crossing-over," Proc. 3rd European Regional Conf. Electron Microscopy, p. 461. Prague Publ. House Czechoslovak Acad. Sci.

Miller, O. L. (1965). *Nat. Cancer Inst. Monogr.* **18**: 79.

Moens, P. B. (1968). *Chromosoma* **23**: 418.

Moens, P. B. (1969). *Chromosoma* **28**: 1.

Moens, P. B. (1970). *J. Cell Sci.* **7**: 55.

Moses, M. J. (1956a). *J. Biophys. Biochem. Cytol.* **2**: 215.

Moses, M. J. (1956b). *J. Biophys. Biochem. Cytol.* **2**: (Suppl.) 397.

Moses, M. J. (1958). *J. Biophys. Biochem. Cytol.* **4**: 633.

Moses, M. J. (1964). *In* "Cytology and Cell Physiology" (G. H. Bourne, ed.), p. 423. Academic Press, New York.

Moses, M. J. (1968). *Annu. Rev. Genet.* **2**: 363.

Moses, M. J. (1969). *Genetics* **61** (Suppl.): 41.

Moses, M. J., and Coleman, J. R. (1964). *In* "Role of Chromosomes in Development" (M. Locke, ed.), p. 11. Academic Press, New York.

Nebel, B. R., and Hackett, E. M. (1961a). *Naturwissenschaften* **20**: 655.

Nebel, B. R., and Hackett, E. M. (1961b). *Z. Zellforsch.* **55**: 556.
Nebel, B. R., and Coulon, E. M. (1962a). *Chromosoma* **13**: 272.
Nebel, B. R., and Coulon, E. M. (1962b). *Chromosoma* **13**: 292.
Ris, H. (1967). *In* "Regulation of Nucleic Acid and Protein Biosynthesis" (V. V. Konisberger and L. Bosch, eds.), p. 11. Elsevier, Amsterdam.
Ris, H. (1969). *In* "Handbook of Molecular Cytology" (A. Lima-de-Faria, ed.), North-Holland Res. Monogr. Frontiers Biol., Vol. 15. North-Holland Publ., Amsterdam.
Roth, T. F. (1966). *Protoplasma* **61**: 346.
Roth, T. F., and Ito, M. (1967). *J. Cell Biol.* **35**: 247.
Rückert, J. von (1892). *Anat. Anz.* **7**: 107.
Schin, K. S. (1965a). *Z. Zellforsch.* **65**: 481.
Schin, K. S. (1965b). *Chromosoma* **16**: 436.
Sheridan, W. F., and Barrnett, R. J. (1969). *J. Ultrastruct. Res.* **27**: 216.
Smith, P. A., and King, R. C. (1968). *Genetics* **60**: 335.
Solari, A. J. (1964). *Nat. Cancer Inst. Monogr.* **14**: 33.
Solari, A. J. (1969). *Genetics* **61** (Suppl.): 113.
Solari, A. J., and Tres, L. L. (1967). *Chromosoma* **22**: 16.
Sotelo, J. R. (1959). *Z. Zellforsch.* **50**: 749.
Sotelo, J. R. (1962). *In* "Fifth International Congress for Electron Microscopy" (S. S. Breese, ed.), Vol. 2, p. XX-6. Academic Press, New York.
Sotelo, J. R. (1969). *In* "Handbook of Molecular Cytology" (A. Lima-de-Faria, ed.), North-Holland Res. Monogr. Frontiers Biol., Vol. 15. North-Holland Publ., Amsterdam.
Sotelo, J. R., and Trujillo-Cenóz, O. (1958). *Exp. Cell Res.* **14**: 1.
Sotelo, J. R., and Trujillo-Cenóz, O. (1960). *Z. Zellforsch.* **51**: 243.
Sotelo, J. R., and Trujillo-Cenóz, O. (1961). *Pathol. Biol.* **9**: 762.
Sotelo, J. R., and Wettstein, R. (1964a). *Experientia* **20**: 610.
Sotelo, J. R., and Wettstein, R. (1964b). *Chromosoma* **15**: 389.
Sotelo, J. R., and Wettstein, R. (1965). *Nat. Cancer Inst. Monogr.* **18**: 133.
Sotelo, J. R., and Wettstein, R. (1966a). *Chromosoma* **20**: 234.
Sotelo, J. R., and Wettstein, R. (1966b). *Nat. Cancer Inst. Monogr.* **23**: 77.
Sotelo, J. R., and Wettstein, R. (1969). *Genetics* **61** (Suppl.): 53.
Sotelo, J. R., and Wettstein, R. (1971). To be published.
Stern, H., and Hotta, Y. (1969). *Genetics* **61** (Suppl.): 27.
Taylor, J. H. (1965). *J. Cell Biol.* **25**: 57.
Taylor, J. H., Woods, P. S., and Hughes, W. L. (1957). *Proc. Nat. Acad. Sci. U.S.* **43**: 122.
Watson, M. L. (1952). Atomic Energy Project (unclassified). The University of Rochester.
Westergaard, M., and von Wettstein, D. (1966). *C. R. Trav. Lab. Carlsberg* **35**: 261.
Westergaard, M., and von Wettstein, D. (1970). *C. R. Trav. Lab. Carlsberg* **37**: 239.
Wettstein, R., and Sotelo, J. R. (1965a). *J. Ultrastruct. Res.* **13**: 367.
Wettstein, R., and Sotelo, J. R. (1965b). *Chromosoma* **17**: 246.
Wettstein, R., and Sotelo, J. R. (1967). *J. Microscopie* **6**: 557.
Whitehouse, H. L. K. (1967). *J. Cell Sci.* **2**: 9.
Wilson, J. Y. (1959). *Genetica* **29**: 290.
Wolstenholme, D. R., and Meyer, G. F. (1966). *Chromosoma* **18**: 272.
Woollam, D. H. M., and Ford, E. H. K. (1964). *J. Anat.* **98**: 163.

REVERSIBLE STABILIZATION OF LABILE MICROTUBULES IN THE RETICULOPODIAL NETWORK OF *ALLOGROMIA*

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I. Introduction

Following Leidy's (1879) elegant description of complex streaming in the weblike reticulopodial network of the fresh water rhizopod *Gromia*

terricola, the motility of these and related sarcodines with reticulopodial networks has been an object of fascination and considerable study on the part of many investigators. Observations on marine foraminifera with similar networks by Doflein (1916), Jepps (1942), Sandon (1934, 1944, 1961), Le Calvez (1938), Jahn and Rinaldi (1959), and Rinaldi and Jahn (1964) have more recently been summarized and extended by Allen (1964, 1968).

The most interesting characteristic of reticulopodial movement is the presence of bidirectional streaming in all parts of the network. In this respect particularly, reticulopodia appear to be different from nearly all other pseudopodia (Allen, 1968). However, some filopodia from marine amoebae show bidirectional streaming and are capable of transforming into lobopodia and pharopodia as well (Allen and Drum, 1969).

Another characteristic of movement in reticulopodia is that "strings" of particles follow one another at nearly identical velocities, and some strings move at higher velocities that are nearly multiples of the lowest velocity observed. These facts led Allen (1964) to postulate a "piggy-back" mechanism operating in "layers" of some kind of linear elements in the individual reticulopodial strands.

The obvious next step has been to determine the ultrastructure of reticulopodia and find out what linear elements are present. Early attempts (Allen and Rebhun, unpublished observations; Allen and Thomas, unpublished observations) using conventional electron microscopic fixatives failed to preserve any linear structures of interest. The first published electron micrographs of sections of reticulopodia in *Allogromia laticollaris* revealed mats of "microfilaments" (Wohlfarth-Bottermann, 1961), but they suggested a number of ultrastructural features that were difficult to reconcile at that time with the light microscopic structure and the observed movements. In retrospect, it is now possible to see that inadequate fixation was the probable cause of some of these results. More recently, the studies of Angell (1967) and of Lengsfeld (1969a,b,c) have also shown the presence of loose bundles of microfilaments similar to those found in slime molds (Nagai and Kamiya, 1966; Kamiya, 1968), *Nitella* subcortical fibrils (Nagai and Rebhun, 1966), and many other motile structures. At the same time, pseudopodia in general have acquired a reputation not only for the diversity of linear structures present, but also for revealing different kinds of linear elements when fixed by different methods. Hedley *et al.* (1967) demonstrated not microfilaments but microtubules in *Shepheardella taeniformis*, but noted that fixation was a significant problem in these pseudopodia, just as Tilney *et al.* (1966) had found in the heliozoan *Actinosphaerium nucleofilum*. The same authors (Hedley and

Wakefield, 1969) failed to find microtubules in the reticulopodial network of *Gromia oviformis* and expressed doubt that microtubules were present.

Reticulopodia of *Allogromia* are quite rigid and show positive birefringence (Allen and McGee-Russell, unpublished observations). This suggests that linear elements must be present in them in the living state. Instantaneous breakup of such reticulopodial strands into "droplets" on interspecific pseudopodial contact (Allen, 1964) suggested that whatever linear elements are present must be highly labile. The same "beading" response and loss of cylindrical shape of reticulopodia occur on contact with all conventional fixatives. Our aim, therefore, has been to stabilize the living morphology and, hopefully, at the same time to preserve the linear elements of the reticulopodial network by a chemical treatment before fixation. This treatment ideally should be fully reversible, so that the animals can be revived on the one hand, or fixed without distortion in stabilized condition on the other. This aim has been achieved, and the results are reported in this article.

The stabilization treatment also gives us some measure of control over the behavior of the animal (to be discussed). This will enable us, in the future, to "dissect" the short-term events of pseudopodial activity and to determine with some precision what ultrastructural characteristics are specifically associated with different pseudopodial events such as the extension and withdrawal of filopodia, branching, fusion, the fiber $\rightleftharpoons$ droplet transition, and other processes that are poorly understood at present.

II. Materials and Methods

The organism selected for this study was a small "naked" foraminiferan originally isolated and cultured by Dr. John Lee and designated *Allogromia* sp. (strain N. F. Lee). This organism is smaller than *A. laticollaris* (Arnold, 1953). It grows prolifically on 2% agar–sea water plates when a few milligrams of dried yeast are scattered over the plate to encourage growth of mixed bacteria.

A. Preparation of *Allogromia* for Prolonged Microscopic Observation

1. *Simple Double Cover Glass Chambers*

By means of a simple "ring-maker" sealing tool illustrated in Fig. 1, a rectangular, thin and uniform perimeter of hot liquid valap (Vaso-

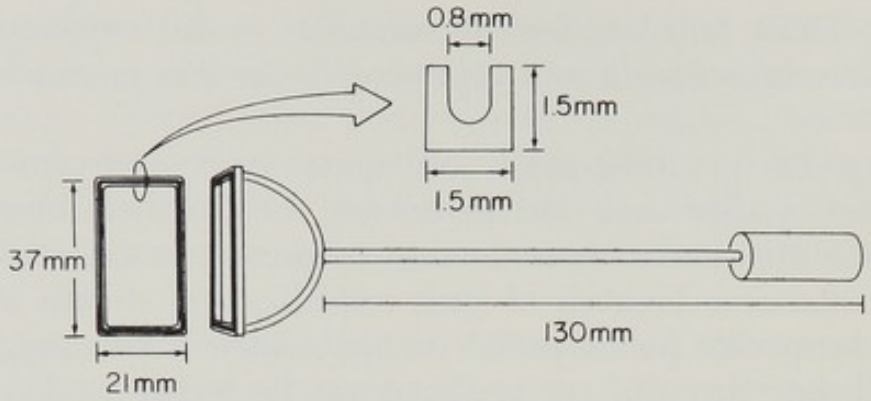

FIG. 1. Line diagram of "ring-maker" sealing tool used with hot valap to form uniform layers of wax as sealing rings around the edges of 22 × 40 mm cover glasses. The profile indicated in the enlarged cross-sectional drawing aids in retaining a standard quantity of wax mixture for transfer to the cover glass, even after the tool has been thoroughly drained of excess valap.

line/paraffin/lanolin, 1:1:1 by weight) was laid down near the edge of a 22 × 40 mm cover glass and in the center of a larger 22 × 60 mm cover glass. The use of the sealing tool ensures that the two perimeters of valap are of closely similar dimensions and thicknesses. A suspension of animals in "Instant Ocean" (Aquarium Systems, Inc., Wickliffe, Ohio) was prepared rapidly by placing one or two drops of liquid onto the surface of an agar culture plate and gently rubbing off the surface fauna. Otherwise, selected animals were detached from the surface with a fine glass probe and rolled across the surface of the agar until a group of about 6–9 had been assembled. This latter method results in preparations which contain fewer bacteria and therefore are superior optically; if the bacterial population is too high, an excessive amount of light scatter reduces contrast and impairs the image quality in phase-contrast or interference microscopy.

The crude suspension of animals was either sampled with a Pasteur pipette, or a selected group of animals was lifted with a micropipette

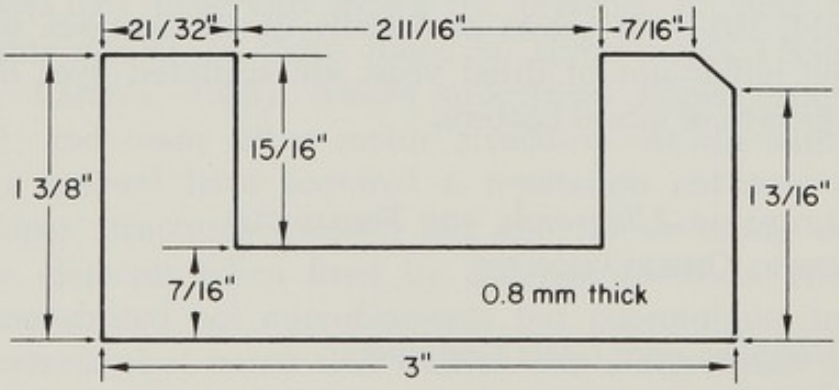

FIG. 2. Line drawing of simple brass holder for support of double-coverslip observation chamber (after A. Bajer, personal communication).

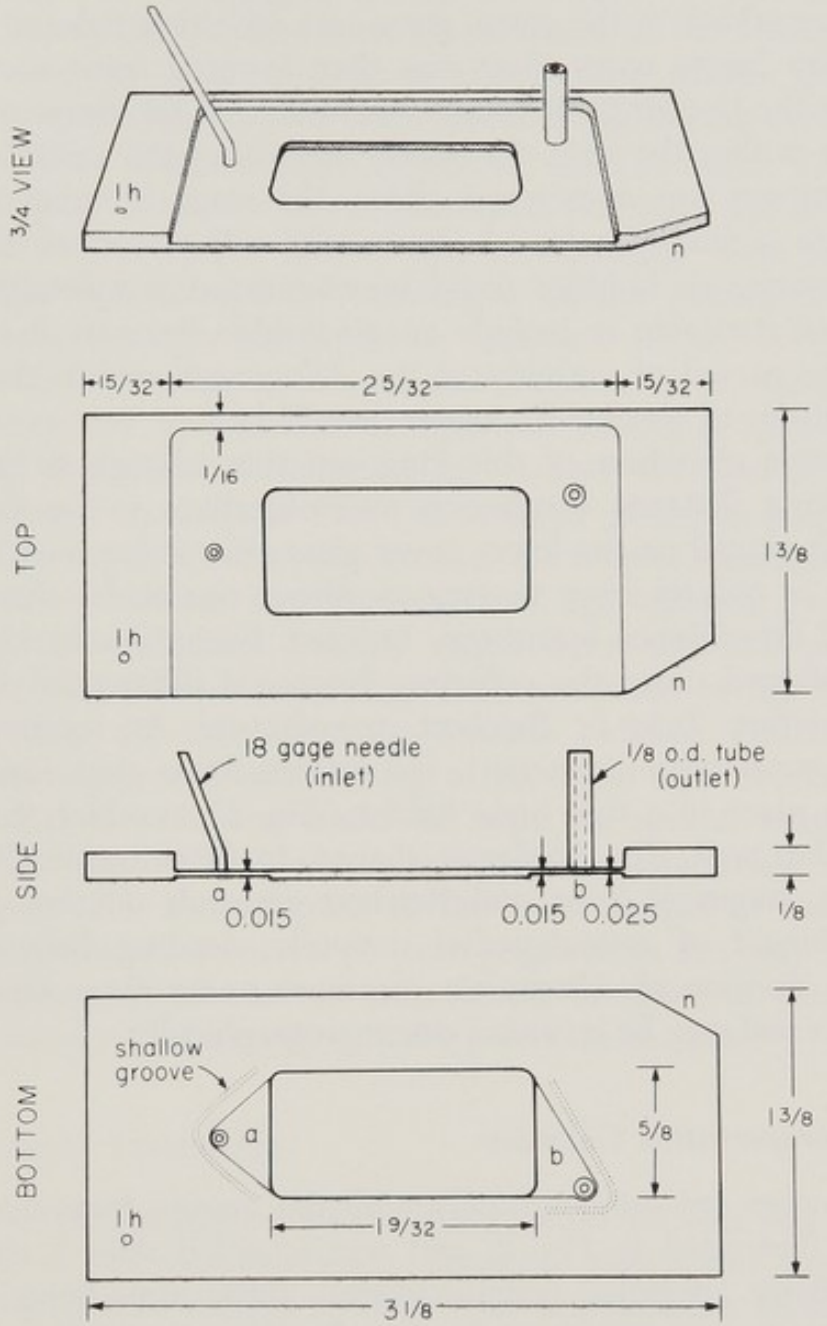

FIG. 3. Line drawing with dimensions showing the configuration and size of a stainless steel microperfusion chamber for combined light and electron microscopical studies. Dimensions are shown in inches. At (a) and (b) note the shallow fan-shaped depressions spreading out from the inlet tube, and narrowing down to the exit tube, which are formed when the bottom cover slip is sealed into place. The positions of inlet and exit tubes are necessitated by the physical dimensions occupied by the objectives on the rotating nosepiece of a standard microscope, such as the Reichert Zetopan. At (lh) is shown a locating hole for a securing pin which helps to stabilize the chamber on the stage of the microscope; at (n) the chamber is chamfered to allow the spring-loaded clip of the stage holder to seat securely. The chamber is modeled after Bajer (1968) and has been modified for use with the marine Protozoan *Allogromia*. It is constructed of stainless steel.

and placed in the center of either cover glass. Two drops of Instant Ocean were added to the cover glass, one on either side of the sample droplet. The larger cover glass was then lowered from above in such a way that the two rings of valap coincided, and the drops of sea water spread out within the ring. By gently squeezing the sandwiched cover glasses between two microscope slides, the rings of valap were made to fuse into a waterproof seal. By careful adjustment of the volumes of the droplets, air bubbles could be eliminated completely. However, it was often desirable to include an air bubble, because it significantly adds to the period of viability of the *Allogromia* within the chamber. We have observed survival for longer than 72 hours.

Observation chambers of this kind are thin enough to permit very short working distance condensers and objectives to be used. If the specimen is placed on the lower cover glass after it has been aluminized and given a quarter-wave coating of silicon monoxide, then reflecting differential interference optics may be used. Exceptionally high contrast can be obtained using the reflecting Nomarski differential interference optics of either Zeiss or Reichert manufacture. As recommended by Bajer (personal communication), the double cover glass sandwich may be held in place in a thin brass holder (Fig. 2) to which it is attached with a valap seal. The holder is shaped to fit conveniently onto the microscope stages of Zeiss and Reichert research microscopes.

Establishment of reticulopodial networks, feeding behavior, movement, and division of *Allogromia* continues under these conditions for many hours and may be recorded cinemicrographically.

2. *The Microperfusion Chamber*

The chamber we used routinely with the marine foraminiferan *Allogromia* is illustrated in Fig. 3, and was modeled after a chamber devised by Bajer (personal communication, 1968; Molé-Bajer and Bajer, 1968). It was necessary to make the chamber of stainless steel. Very narrow fan-shaped openings leading from the bases of inlet and outlet tubes were formed by sealing the lower 22×60 mm cover glass onto the underside of the chamber. The fan shape of these openings ensured that a straight curtain of liquid flowed without turbulence across the chamber so that interchanges of liquid perfusate could be made rapidly. The inlet tube must be significantly smaller than the outlet tube in order to maintain relatively constant hydrostatic pressure in the chamber.

The animals were placed in a small droplet onto the center of a 22×40 mm cover glass, inside a rectangular perimeter of valap made with the same sealing tool. The droplet was covered with a film contain-

ing 0.75% gelatin, 0.75% agar, and 2.5% dextrose (w/v) in double-distilled water (Bajer, 1968). Care must be exercised in the choice of agar and gelatin used, in order to achieve strong films of the greatest optical clarity. In this laboratory we have found the following components satisfactory: Difco Purified Agar (certified) (Difco Labs, Detroit, Michigan); Gelatin, Granular, USP, (Matheson, Coleman and Bell, Cincinnati, Ohio); Dextrose (J. T. Baker, Co., Phillipsburg, New Jersey).

The film was formed by drawing a square platinum loop about 1 cm $\times$ 1 cm through the liquid-air interface of a freshly poured hot solution, and allowing it to cool and stabilize for a moment. A certain knack, acquired by practice, ensures the casting of good films on the loop and successful transference to enclose the specimen droplet. A second, larger rectangular film (on a larger platinum loop) was superimposed on top of the first film, so that the droplet containing *Allogromia* was enclosed beneath two thin layers of agar–gelatin. The second film provided the additional stability required for repeated perfusions. Because the films introduced little water compared to the volume of the droplet, they did not upset the osmotic balance. Instant Ocean was immediately pipetted onto the top of the fresh agar overlays to prevent desiccation; preparations could be kept for several hours in a moist chamber. Within 40 minutes many animals reestablished their reticulopodial networks and adapted to the environment of the agar–gelatin enclosure. Numerous coverslips were prepared in this way and inspected by low-power phase-contrast microscopy in order to choose the most active specimens for mounting in the perfusion chamber.

B. Perfusion Technique

A cover glass prepared with suitably active animals was inverted onto the well of the microperfusion chamber, which had previously been filled with Instant Ocean solution. The combination of correct volume and an edge ring of valap on the cover glass ensured a bubble-free system with no overflow. Hot valap was then run onto the edge of the coverslip to fuse with the valap ring and seal onto the metal of the chamber. Several layers of valap ensured a leak-proof seal. Careful handling was necessary to avoid heat damage to the animals during this process, and a period of recovery was allowed for full establishment of normally active pseudopodial networks. Unless the well of the perfusion chamber was filled with liquid to limit the temperature rise, severe damage sometimes resulted. However, with due precautions, fully active normal behavior was observed within a few minutes after sealing the chamber.

The final disposition of the animal within the chamber is illustrated in Fig. 4. The dimensions of the chamber permit all forms of transmission light microscopy using both Zeiss and Reichert research microscopes equipped with interference and phase-contrast optics. A color film demonstrating the technical details of the handling procedures and the observations possible with the microperfusion chamber is available for loan by the authors (McGee-Russell and Allen, 1969).

In order to perfuse the chamber during high magnification–high resolution light microscopy, it first had to be firmly clamped onto the adjust-

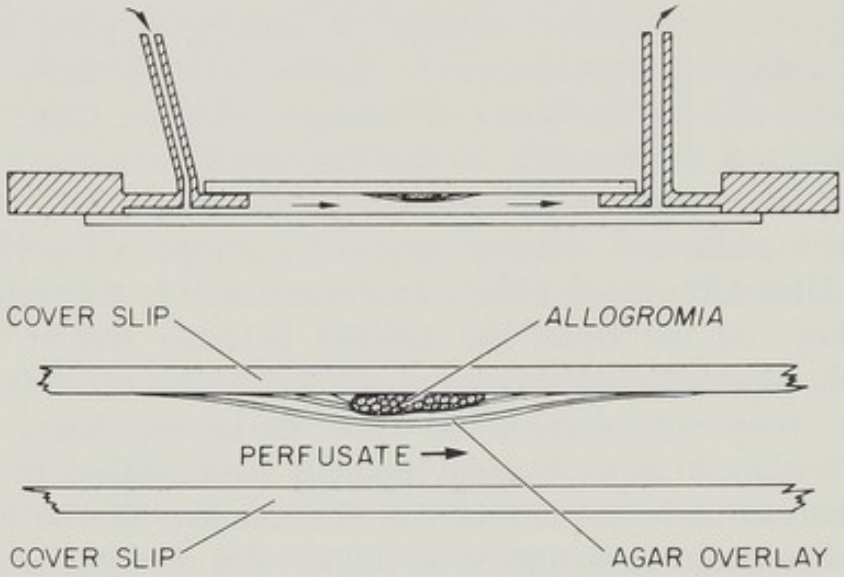

FIG. 4. Line drawing showing the microperfusion chamber during perfusion of an *Allogromia*, isolated beneath an agar overlay. The flow rate indicated by the arrows is calculated to be of the order of 5 cm/sec, so that an exchanging interface between perfusion fluids passes the animal within a few milliseconds.

able stage of the microscope with metal locating pins and sticky tape. An animal was then chosen and centered under the objective. The perfusion system consisted of a 20-ml plastic syringe barrel minus its plunger, equipped with an 18-gage blunt-ended outlet needle. The open syringe barrel was held in a retort stand approximately 19–27 cm above the plane of the microscope stage. The inlet tubing consisted of about 66 cm of Clay-Adams intramedic polyethylene tubing type P.E. 190 (I.D. 0.047 inches, O.D. 0.067 inches), compatible with the exit needle of the syringe and the 18-gage inlet needle of the microperfusion chamber. The outlet tubing (0.115 inches I.D., 0.147 inches O.D., type P.E. 330) had a length of 68–71 cm and was held on the same bench retort stand so that its end dipped conveniently into a 500-ml conical flask as a collection reservoir.

In use, these simple elements were operated in the following way:

(1) The outlet tube was slipped onto the exit tube from the perfusion chamber. (2) The barrel of the syringe was filled with the perfusing medium (initially Instant Ocean). The open end of the syringe was large enough to permit very rapid filling. The liquid flowed down the polyethylene tube, ensuring a bubble-free system. With the liquid medium dropping steadily from the open end of the inlet tube onto a suitable collection pad, the tubing was rapidly slipped onto the inlet needle of the perfusion chamber, again avoiding bubbles. (3) The outflow rapidly rose up the outlet tube, usually filling it steadily without bubbles, until a succession of drops emerged at the end. At this time, when the tubing was filled with a continuous column of liquid, the end of the outflow tube was pushed below the surface of the liquid in the reservoir; this prevented the periodic change of pressure and consequently of microscope focus which occurred through back pressure if drops were allowed to form and drop off the end of the outlet tube. Once this system was set up it permitted a continuous flow through the chamber at a rate of approximately 1 ml every 5–15 seconds. (4) In order to change the composition of the perfusate rapidly, the level in the syringe barrel was permitted to fall to 1 ml or less, and then a large excess of 20 ml or more of the new solution was added very quickly to keep the dilution factor small.

Model experiments with concentrated dye solutions showed that there was a sharp discontinuity between the border of the dye and the clear fluid previously filling the chamber. From the rate of fall of the fluid in the entrance syringe and the cross-sectional areas of the syringe and the central third of the chamber through which a "shaft" of dye races, it was computed that the interface travels at a velocity of 5 cm/sec (50,000 μ/sec). Thus, an *Allogromia* would be passed by the interface within a few milliseconds. Diffusion through the gelatin–agar films would hardly be expected to lengthen appreciably the interval between arrival of the interface and complete mixing in the vicinity of the reticulopodial network.

Most of our work to date has been carried out using a Reichert Zetopan stand equipped with "Anoptral" phase-contrast (Wilska, 1953, 1954), special high absorption positive phase-contrast, and Nomarski differential interference optics (for details, see Padawer, 1968; Allen *et al.*, 1969). The cinemicrographic system was a wall-mounted Arriflex 16 mm camera equipped with a Kine Konnex viewing head and Reichert adapter. Photometry was carried out with a Photovolt model 512, and film records were made with British Kodak or Recordak High Contrast Copy Film, or else with Kodak Ektachrome Commercial Color Film (type 7255).

Because of the rapidity with which events such as beading, breakup of pseudopodia, and stoppage of streaming on stabilization occur, it has been necessary to make cine records at framing rates of at least 16 frames/second.

C. Combined Light and Electron Microscopy of Mapped Reticulopodia

The methods discussed above were developed in order to permit exact correlation between the living activity of individual animals and the fixed ultrastructure studied in the electron microscope. In order to locate the precise region to be studied electron microscopically, and in order to orient the plane of sectioning properly, it was necessary to have thin flat embeddings, the surfaces of which had optical quality. Removal of the cover glass from the Epon block after conventional carbon or collagen coating was not always successful. Therefore, we used cover glasses pretreated with the fluorocarbon spray MS-122 (marketed by Miller and Stephenson Co. of Danbury, Connecticut). The spray was applied to one side of the cover glass on which the specimen was to be placed, and the glass was then thoroughly polished with paper tissue until optically clear. Enough MS-122 remained so that later the cast block separated cleanly from the cover glass, and the surface of the block was of high optical quality because it was molded by an optically finished surface.

After specimen fixation, the chamber was perfused with buffer followed by dehydrating agents such as ethyl alcohol. We normally terminated observations in the buffer rinse, removed the cover glass from the chamber, and carried out electron microscopical processing. For dehydration, the cover glass was transferred from petri dish to petri dish with tweezers. Since the least disturbance of the preserved reticulopodial network occurred with the shortest processing times, we dehydrated with a stop watch as timer, allowing 10 seconds in each of two dishes of the following concentrations (% v/v) 20, 30, 50, 70, 80, 90, absolute ethanol; 1,2-epoxy propane; followed by single immersions in 1,2 epoxy propane-Epon C (1:1); and Epon C (Luft, 1961). Once the specimen reached the mixture of propylene oxide and resin (PO/Epon C 1:1), it was regarded as stable and was left in this mixture or in the final catalyzed Epon C for many minutes.

Blocking out was performed by placing one or two drops of fully catalyzed Epon C onto an MS-122 treated and polished glass slide between two glass spacers, also coated. The thickness of the glass spacers

was adjusted to give the best combination of mechanical stability during sectioning, and maximum visibility of the organism with phase or interference optics. For spacers, we used two piles of three or four No. 1 cover glasses (or for thick wafers two pieces of glass slide). The infiltrated cover glass preparation was then inverted onto the drops of Epon and allowed to settle parallel to the spacers. It was not usually necessary to apply weights.

In a flat embedding of this kind the organism lay just at the surface of the plastic, or within a few microns of it; since the wafer was thin and the surfaces of the plastic on both sides were optically perfect, high resolution light microscopy could be carried out on any portion, as shown in Figs. 5 and 6. In order to study the pseudopods ultrastructurally, the most useful plane of section was transverse to the long axis, i.e., at right angles to the plane of the flat embedding (Fig. 6). The wafer block was held satisfactorily in the flat embedding chuck of the Porter-Blum MT 2 ultramicrotome; however, this resulted in some mechanical damage to the surface of the plastic, especially undesirable when it was the specimen surface. In order to section parallel to the embedding plane, individual animals were cut out of the wafer and mounted on the ends of blank blocks.

During sectioning and observation in the electron microscope an additional difficulty was experienced with the wafer embeddings. Because the specimen lay at the extreme edge of the section, when the plane of section was transverse to the plane of embedding the specimen was effectively unsupported by plastic on one side. This made it necessary to use carbon-coated or formvar-carbon-coated grids for mounting these sections, with a consequent loss of contrast. This could be avoided by re-embedding the specimen so that its surface was below a further layer of Epon plastic, cast in the same way against a coated glass slide to give an optically flat surface. We devised a procedure using an Epon–Araldite mixture for this purpose, which had the significant advantage that it provided a built-in interface visible in the electron microscope; this acted as a guiding landmark and reference, capable of leading us precisely to the desired plane in the preparation. In sections cut from such double-wafer embeddings the specimen was bounded and fully supported by plastic on both sides, signposted by the interface, and the sections could be mounted on uncoated grids for maximum contrast and resolution in the electron microscope. The technical details of this double-wafer embedding procedure will be published by Bender *et al.* (1970).

Either the single or the double-wafer preparation may be inspected with phase-contrast or interference optics at any time during sectioning,

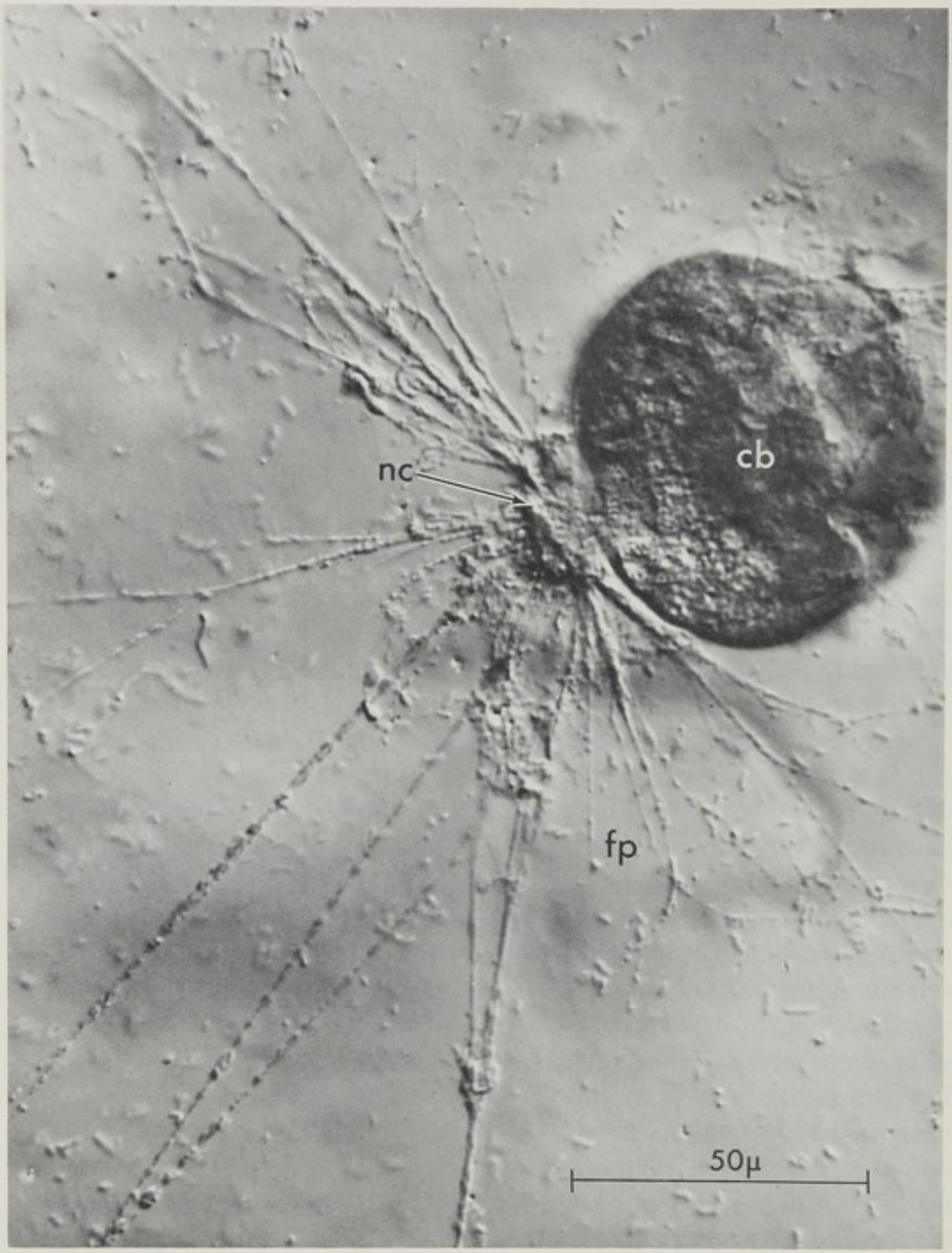

FIG. 5. Small preserved *Allogromia* photographed in Epon plastic wafer by Nomarski transmission differential interference optics after treatment as follows: 38% $MgCl_2$; 6.5% glutaraldehyde in cacodylate buffer pH 7.4; cacodylate buffer rinse at same pH; 1% osmium tetroxide in cacodylate buffer pH 7.4; cacodylate buffer rinse (all single treatments of 10 seconds each); 20%, 30%, 50%, 70%, 80%, 90% (all double treatments of 10 seconds each); absolute ethanol (2 minutes); propylene oxide (2 × 30 seconds); propylene oxide–Epon C mixture (prolonged); Epon C (prolonged) and embedded out as described in the text. The linear form

in order to monitor position with respect to previously mapped features (see Figs. 5 and 6). With care, the level of sectioning may be controlled to the limit of resolution of the light microscope. We have carried out a very precise study of individual pseudopods of selected animals, using the light microscope to record by cinemicrography the living activity of fully identified pseudopods both before and during chemical fixation, and the electron microscope to discover ultrastructural details of selected regions of these same pseudopods. The electron microscopes used were the A.E.I. EM6B and the Phillips EM300. Accelerating voltages were 40 and 50 kV. Sections were cut on Reichert OMU-2 and Porter-Blum ultramicrotomes with diamond knives and stained with 2% aqueous uranyl acetate at 37° C.

III. Results

A. Normal Behavior of *Allogromia* in a Perfusion Chamber

The general characteristics of movement in *Allogromia* sp. (strain N. F. Lee) were described by Allen (1964). We have observed that behavior is completely normal in the observation chambers. New pseudopodia are characteristic filopodia emerging as stiff rods, with rounded droplets of hyaline cytoplasm at the extreme tip (the "bouton terminale" of Le Calvez, 1938). These filopodia extend from an oral opening where the theca of the animal forms a "neck region" (Figs. 5, 6, 7, and 8). The theca appears to conform closely to the shape of the cell body, which often exhibits "lurching (amoeboid) movements." We have designated cytoplasm which emerges from the oral opening as "neck cytoplasm." There is a marked difference between the organellar composition and texture of the cell body cytoplasm (enclosed within the theca) and the neck cytoplasm (Figs. 7 and 8, Table I). The neck region may constitute a "transformation zone" interposed between the cell body cytoplasm and the cytoplasm of the reticulopodial network; in some specimens the ultrastructure is also suggestive of this. A thick neck of cytoplasm passes out of the oral opening in the theca and terminates in a large rounded mass of cytoplasm from which numerous filopodia and reticulopodia radiate (Figs. 5 and 6).

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and naturally variable width of filopods down to the finest, has been fully preserved. Particles within the finest pods frequently appear broader than the diameter of the filopod itself. This lifelike appearance should not be confused with the grossly beaded configuration produced by a damaging fixation. nc = neck cytoplasm; cb = cell body; fp = filopodia. Magnification approximately 1180×.

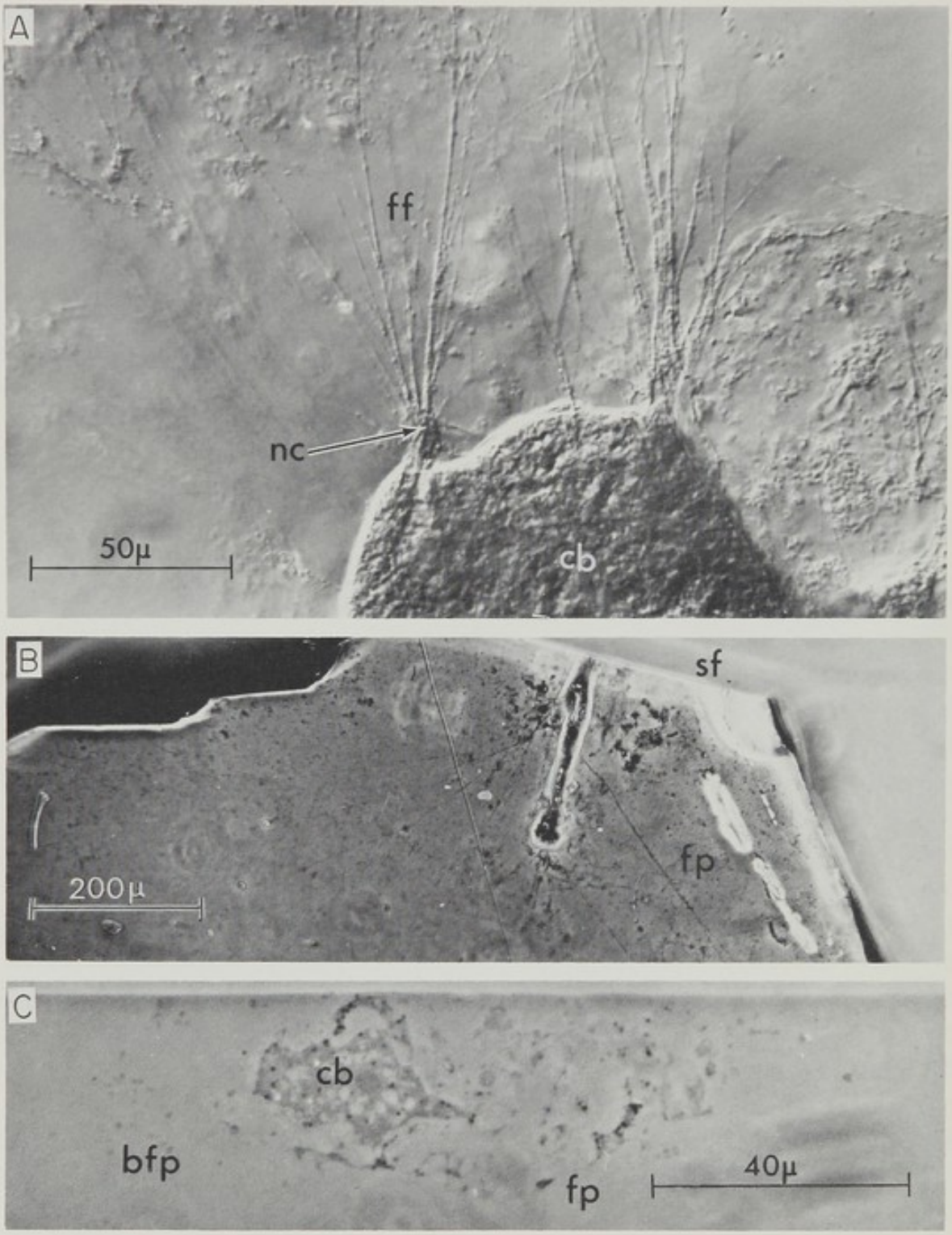

FIG. 6A. Transmission differential interference micrograph of one end of a multipolar *Allogromia* showing in this region two large oral zones with emergent fans of filopodia. At (nc) note the accumulation of neck cytoplasm at the entrance to the oral zone. This micrograph is one of a through focus series. Such series are required in order to reconstruct the full three-dimensionality of the network. Treatment and fixation were the same as given in Fig. 5. nc = neck cytoplasm; cb = cell body; ff = filopodial fan. Magnification approximately 792×.

FIG. 6B. Bipolar *Allogromia* photographed by positive phase contrast during sectioning, without removal from the chuck of the microtome. Precise orientation

In life, the cytoplasm of the reticulopodial network is extremely dynamic and is continually changing its form as pseudopods thin and thicken, fuse and separate, flatten into webs which at every point show rapid bidirectional streaming of particles. This bidirectional streaming is a locally semiautonomous process, the control of which must in large part lie within the local area of pseudopod under observation. Bidirectional streaming continues in elements of the network totally isolated from the cell body and its many nuclei, for periods up to several hours (Jahn and Rinaldi, 1959; Allen, 1964). However, the cell body cytoplasm and nuclei are a fundamental source of either energy substrates or of some essential control, since the isolated pieces of network eventually "run down" and can only be reactivated by an infusion of fresh cytoplasm from the cell body (Allèn, 1964). As will be described later, this process has now been brought under some degree of experimental control, and we hope to analyze this phenomenon in greater detail at the ultrastructural level (see also Lengsfeld, 1966b).

After *Allogromia* are explanted into the observation chamber, they rapidly assume mono-, bi-, or tripolar forms and eventually undergo division into two or more smaller animals. The fact that cells divide in the chambers and continue to live normally for many hours is indicative of their healthy condition.

B. Response to Chemical Fixatives and Other Treatments

The aim of chemical fixation is the preservation of form and ultrastructure in as lifelike a condition as possible. However, the active reticulopodial network of *Allogromia* is, in our experience, the most difficult biological object in which to achieve this aim. By contrast, the cell body and its enclosed cytoplasm are comparatively easy to prepare by fixation; various conventional fixatives (glutaraldehyde, formaldehyde, osmium textroxide, and aldehyde primary fixation followed by osmium postfixation) all give moderately satisfactory preservation

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is maintained. This is organism k FA 82, level of sectioning 21. sf = sectioning face; fp = filopodia. Magnification approximately 169×. Treatment and fixation were the same as for Fig. 5.

Fig. 6C. Positive phase-contrast image of the same animal shown in Fig. 6B. (organism k FA 82) However, this is a thick (1 μ) Epon section unstained, taken at the same level 21 as in Fig. 6B. cb = cell body; fp = filopodia; between cb and fp are minute profiles of transversely sectioned bacteria and filopods. Many of the filopods are narrower in diameter than the adjacent bacteria [as at bfp]. Magnification approximately 1273×.

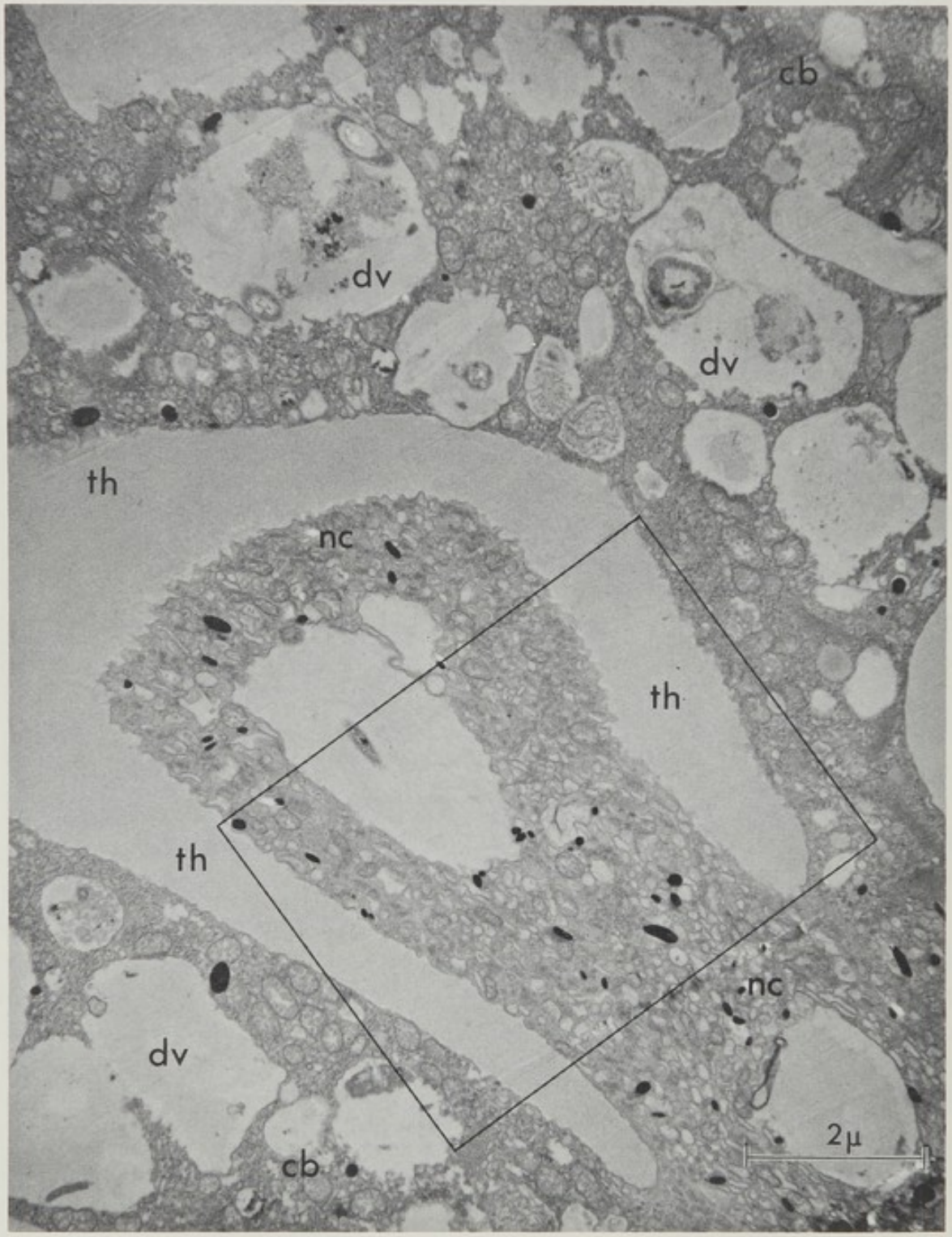

FIG. 7. Electron micrograph of Epon section passing through the neck region of *Allogromia*. Primary osmium tetroxide fixation (without stabilization). Note the slightly darker contrast in cell body cytoplasm (cb) due to a large population of ribosomes between abundant digestive vacuoles (dv), as compared with the lower contrast of neck cytoplasm (nc) packed with numerous smooth membrane-bounded small vesicles (vs). The theca (th) encloses the annular promontory of neck cytoplasm. The narrow black line outlines the area shown at higher magnification in Fig. 8. Magnification approximately 17,500×.

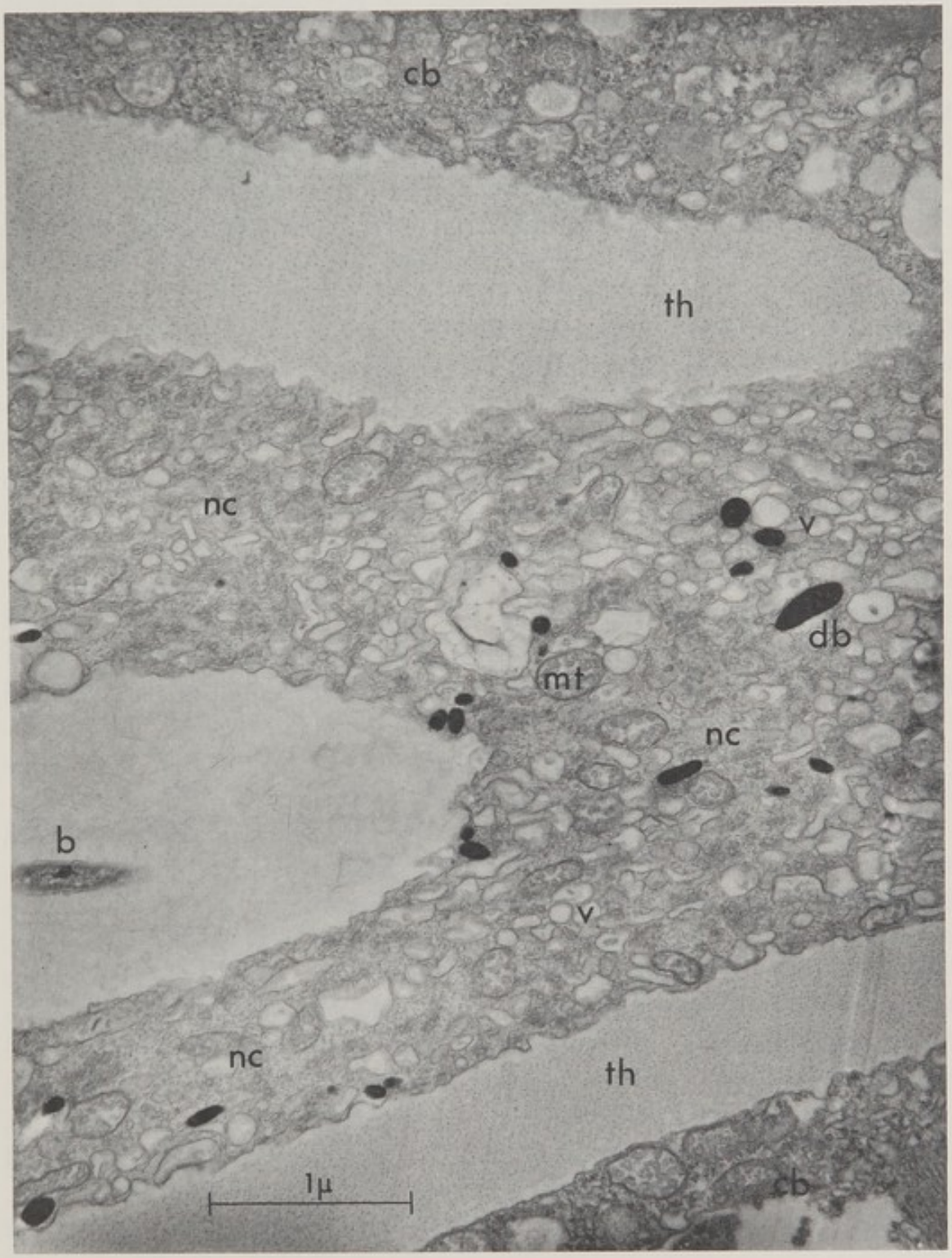

FIG. 8. Higher magnification electron micrograph of area outlined in Fig. 7. Note the abundance of ribosomes in the cell body cytoplasm (cb), numerous small membrane-bound vesicles (v) in the neck cytoplasm (nc), finely fibrillar matrix of the theca (th), dense bodies (db), mitochondria (mt), bacterium (b), and general absence of transverse or longitudinal profiles of microtubules. Contrast this image with Fig. 9B, also of neck cytoplasm, but after stabilization treatment. Magnification approximately 40,000×.

of the cell body cytoplasm, and even of the cytoplasm of the neck region.

Attempts have been made to fix the reticulopodia with and without sea water, with and without added calcium, with most conventional buffer systems at different pH levels, with and without added sucrose over a range of concentrations, for each of the conventional fixatives. In addition, simple potential fixatives were tested, including solutions of heavy metal ions such as uranyl acetate, mercuric chloride, potassium dichromate, picric acid, chromic acid, and also the simple organic solvents alcohol, acetone, and chloroform. In every case the reticulopodial network responded to the arrival of the chemical agent by immediate and violent distortion and disruption. The pseudopods "beaded" instantaneously, and they frequently snapped and broke apart. Even the very thickest of pseudopodia close to the neck region, forming a "main trunk" in the network, showed this severe damage. However, some faint semblance of linearity was retained in the thickest strands, which under uncritical examination might suggest some degree of preservation of the reticulopodial network. When the moment of fixation is observed or recorded cinematographically with an adequate phase-contrast or interference microscope, it is easily seen that conventional fixatives fail utterly in preserving the lifelike form and appearance of the reticulopodial network. On the other hand, the cell body, theca, and to some extent the neck cytoplasm are reasonably well preserved.

The beading response is characteristic when the chemicals are applied rapidly in conventional fixative concentrations. However, if the concentration of the chemical is too low, the animal has sufficient time to respond by "reeling in" the entire network through the oral opening. This seems to be a normal escape reaction, and is evinced in response to almost *any* rapid change in an equilibrated environment (even a temperature change of Instant Ocean perfusate, in the normal physiological range). If the temperature is raised too rapidly, again the pseudopods show the beading reaction. This can happen, for example, if organisms are mounted carelessly in microperfusion chambers and suffer from heat diffusing away from the hot valap sealing compound. With a dilute chemical solution and a normal fast flow rate, the animal may partially succeed in withdrawing the extended network, but the remainder then suffers damage and becomes beaded.

The severe visible damage produced by *every* fixative combination motivated us to investigate anesthetizing or inactivating solutions applied to the animal before the fixative. Much effort was devoted to an investigation of carbon dioxide as a potential anesthetic because of its action on slime molds (Kamiya and Nakajima, 1955). However, it too produces

beading, without any sign of inactivation or cessation of movement. So do chloroform and tricaine methane sulfonate (M.S. 222), which were also tested extensively.

Palay *et al.* (1962) and many others demonstrated some benefits of added calcium for electron microscopical fixation. Therefore, solutions of calcium, strontium, and barium salts (chloride, sulfate, and phosphate) in Instant Ocean, from concentrations of 0.5% up to saturation, were tested for their effect upon living networks. With all except one, the animal usually showed either the withdrawal-escape reaction or the beading response to damage. However, in highly concentrated calcium chloride solutions in Instant Ocean, the thicker pseudopods retained some degree of straightness.

C. Stabilization Treatments

1. *Reversible Stabilization by Low Concentrations of Magnesium Chloride*

Magnesium chloride is a well-known anesthetic for marine invertebrates, and to maintain isotonicity it has been used as a 7.5% w/v solution of the hexahydrate mixed in equal proportions with sea water. Magnesium is also known to act antagonistically to calcium in many physiological systems (Heilbrunn, 1952). Therefore, to parallel our test with Instant Ocean enriched with calcium chloride, Instant Ocean was enriched to double its isotonic strength by the addition of 7.5% w/v $MgCl_2 \cdot 6H_2O$. We tested solutions ranging from this concentration up to 38% in Instant Ocean.

When a perfusate of Instant Ocean is followed by a 7.5% solution of magnesium chloride in Instant Ocean, the animal immediately initiates the withdrawal-escape reaction, and the whole of the network starts withdrawing into the theca through the oral opening. Bidirectional streaming continues locally, but the overall movement of the network is inward. Under the action of the 7.5% $MgCl_2$ the withdrawal reaction may be subdivided into four distinct phases: (1) overall inward movement of the net; (2) progressive thinning of the pseudopods; (3) relaxation and loss of tension in the thinned pseudopods, which is evinced as a flaccidity of the strands immediately before the final stage; (4) stabilization, wherein all movement ceases. During stages 1–3, movement of individual particles continues in both directions, although overall inward movement of the network continues in phases 1 and 2. Only in stage 4 does all activity cease. The whole sequence of events takes up to 20 seconds or longer.

The specimen may be maintained in stage 4 for minutes, by continued perfusion with 7.5% $MgCl_2$ in Instant Ocean. However, if the $MgCl_2$ perfusate is replaced with a normal medium of Instant Ocean, a remarkable series of events occurs as soon as the Instant Ocean reaches the stabilized pseudopodia. Motion restarts virtually immediately. However, it is not local and autonomous bidirectional streaming, but the "reeling-in" motion of the entire reticulopodial network, which is reactivated. A brief time later, bidirectional streaming patterns are reestablished. It appears that control of the withdrawal response is directly inactivated by the magnesium chloride treatment, and that as soon as this is washed away the physiological behavior initiated earlier to withdraw the reticulopodial network continues without modification. This withdrawal response may go on virtually to completion, or if a particularly well-developed reticulopodial network is present, the next stage of regrowth and reestablishment may be observed in those pseudopods which do not completely withdraw. The reactivated bidirectional streaming appears to contribute "fresh" cytoplasm endowed with influences that specify outgrowth from the mass of cytoplasm in the cell body to the residual pseudopods. One can observe a wave of such fresh cytoplasm flowing out into the very fine threadlike pseudopods which may be all that remain of the original network. Such a wave is led by a bleb or droplet similar to the hyaline droplet at the end of a new pseudopod, and behind it all parts of the regrowing pseudopod show active bidirectional streaming. If the network is completely withdrawn into the cell body, then completely new pseudopods grow out from the oral zone and reestablish a new network within several minutes. It is possible to repeat this cycle many times with the same animal by alternate perfusion with Instant Ocean versus 7.5% $MgCl_2$ in Instant Ocean, and thus, by controlling the behavioral response of the animal, to film and study these two processes.

2. *Instantaneous Stabilization by High Concentrations of Magnesium Chloride*

The reversibility of the stabilization achieved with a lower concentration of $MgCl_2$, and the control which it affords over the behavior of the animal, is very valuable. However, it does not represent an instantaneous stabilization of the living form of the network, and if a conventional fixative is used on such an animal beading still results. We therefore tested concentrations of $MgCl_2$ solutions in Instant Ocean from 7.5% up to 38% (38 gm of $MgCl_2 \cdot 6H_2O$ in 100 ml Instant Ocean) in graded steps of 5%, for their effect upon the living networks as observed

with high-power phase-contrast microscopy. The principal parameter of change in the response of the animal was the time interval during which the withdrawal response continued before stabilization was achieved.

At and above a concentration of 30% $MgCl_2$ in Instant Ocean, there is virtually instantaneous cessation of all movement in all parts of the reticulopodial network, with no further changes of form. Even the finest filopodia remain unbroken and undistorted; there is no beading response. Because we had indications that the process is slightly more rapid at higher concentrations, we have routinely used 38% $MgCl_2$ for most of our recent experiments; nevertheless, the critical threshold for rapid and immediate stabilization lies at the 30% level.

When such a preparation is treated directly with a conventional fixative, such as glutaraldehyde in Instant Ocean or in cacodylate buffer, there is no change in the network, and the entire animal is preserved for ultrastructural analysis with a lifelike appearance. The remarkably lifelike preservation which may be achieved by this procedure is illustrated in Figs. 5 and 6A, both of which are photomicrographs of animals embedded in Epon after stabilization, fixation in glutaraldehyde, and postfixation in osmic acid. The quality of the preservation is such that it is impossible without prior knowledge for the authors to distinguish between a Nomarski interference photomicrograph of a living animal and of one of these animals embedded in plastic.

The stabilization medium has approximately nine times the osmotic pressure of sea water, and it also upsets the ionic balance that plays such an important role in regulating physiological activity. For that reason, we wished to determine whether the stabilization effect is due to osmotic pressure alone, ionic imbalance, a specific effect of magnesium ions, or a combination of these and other factors. To test whether osmotic pressure alone would cause stabilization, networks were perfused with sodium chloride at nine times the osmotic pressure of sea water (4.5 molal). Thinning of pseudopods was very evident and streaming apparently stopped, but the pseudopods became so highly refractile that details could not be followed. The cell body distorted severely. To test whether ionic balance was important in stabilization, calcium-free sea water and isosmotic magnesium chloride (0.34 molal) were tried. Neither solution had a rapid stabilizing effect. Calcium-free sea water gave immediate beading, but the animal adapted and extended a new network. Isosmotic magnesium chloride in distilled water gave almost complete withdrawal of the network, without disruption of the linear fiber appearance of the pseudopods. However, if any pseudopods remained outside the cell body, which was rare, streaming stopped; conse-

quently, there was some stabilization. Magnesium-free sea water affected the animals very little, and indeed, appeared to stimulate growth of new pseudopods. Increasingly hypertonic concentrations of magnesium chloride in distilled water caused more and more rapid onset of stabilization during withdrawal, but even the highest concentration tried (38%) was not as effective as Instant Ocean to which high magnesium chloride had been added.

It can be concluded that hypertonicity, high magnesium concentration, and the presence of other sea water ions are all important factors in instantaneous stabilization. No reversible recovery has been obtained with alternative solutions.

3. *Reactivation and Recovery of Stabilized Animals*

It might be expected that 38% magnesium chloride in sea water should be aphysiological and damaging to the animal; remarkably enough, this is not true. When followed immediately by a wash with Instant Ocean, the extended and stabilized reticulopodial network immediately shows beading damage and disrupts, but the whole animal and the cytoplasm within the theca apparently remain undamaged; after several hours in Instant Ocean, such a treated animal fully recovers and extends a normal reticulopodial network. Either the theca, the cell membrane, or both have a significant protective function in this response, and we regard the mass of cytoplasm within the theca as significantly different physiologically from the cytoplasm comprising the network.

D. Ultrastructural Observations

After conventional fixation with osmium tetroxide, normal cellular elements may be observed in the cell body and neck cytoplasm (Figs. 7 and 8; Table I). In the cell body these include nuclei and typical cytoplasmic organelles such as ribosomes, endoplasmic reticulum, digestive vacuoles, Golgi zones, and abundant small, smooth membrane-bounded vesicles. Rough endoplasmic reticulum is not noticeable in either cell body cytoplasm or in neck cytoplasm. The cell body has a notable abundance of ribosomes, and Golgi zones are numerous, probably associated with the digestive function of the cell body as well as with the active membrane synthesis which must be required for development and maintenance of the reticulopodial network.

The cytoplasm of the neck region is easily identifiable in primary osmium-fixed sections because of its large population of smooth

TABLE I
RELATIVE NUMBERS (COUNTS PER 10 μ^2) OF INCLUSIONS IN BODY AND NECK CYTOPLASM OF *Allogromia*

	Smooth membrane vesicles	Mitochondria	Dense bodies	Ribosomes	Polysomes	Amorphous dense regions
Body cytoplasm	97	12	2	>325	54	5
Neck cytoplasm	452	20	27	0	0	45

membrane-bounded vesicles, and the almost total absence of ribosomes (Figs. 7 and 8; Table I). Both of these properties often characterize a "stream" of neck cytoplasm extending some distance into the cell body from the neck zone, which then merges imperceptibly with the characteristic cell body cytoplasm. The neck cytoplasm often assumes a cylindrical shape as it passes through the oral opening, so that it has a central space surrounded by an annulus of cytoplasm (Figs. 7 and 8).

After primary osmium fixation neither the neck region, nor the cell body, nor the pseudopods show any pronounced linear elements such as microtubules. However, after rapid stabilization with $MgCl_2$ followed by conventional fixation with glutaraldehyde and postosmication, nearly every section of the network shows the presence of microtubules oriented along the long axis of the pseudopodium (Figs. 9 and 10). In the neck cytoplasm massed bundles of microtubules occur (Fig. 9); these may adopt a somewhat sinuous course through the neck zone, and there does not appear to be any particular order or packing pattern to their distribution, inasmuch as they may be either widely separated or in close juxtaposition. The long axes of the microtubules are usually parallel to the long axis of the neck cytoplasm. It is not yet clear what happens at the points where the cytoplasm divides to give rise to individual pseudopodia, but within pseudopodia the microtubules usually occur as a restricted group of elements in the cytoplasm contained by the single plasmalemma surrounding each pseudopodium (Fig. 10). The microtubules in pseudopodia may lie so close together that they touch (arrow, Fig. 10); however, as in the neck region, they may also be well separated. No sign of cross-bridging has been detected as yet (however, see Hedley *et al.*, 1967).

Within the pseudopods, in addition to the microtubules, there is normally a somewhat amorphous background density of material lying between typical organelles such as mitochondria and unit-membrane bounded vacuoles. Both mitochondria and small, similarly sized, dense

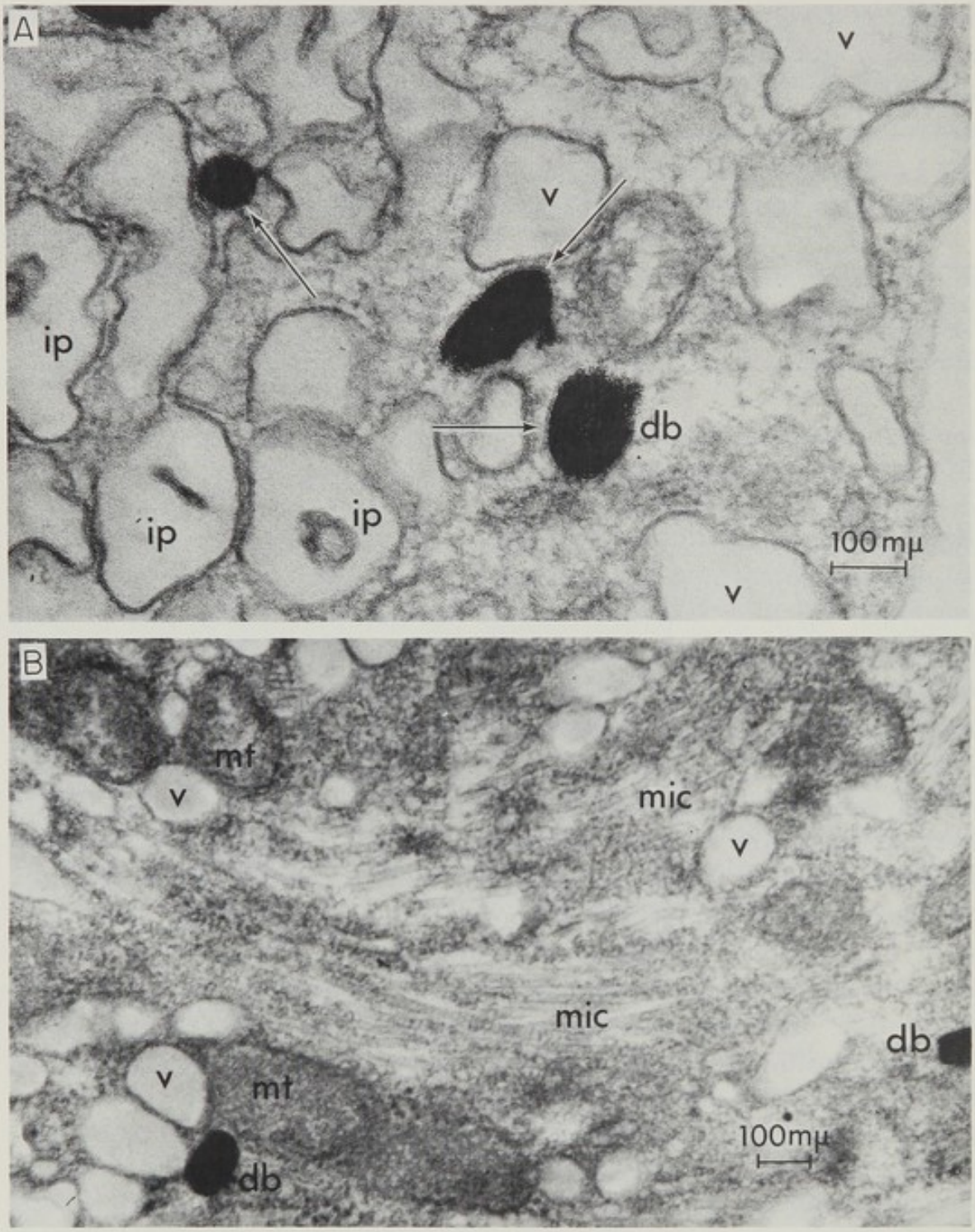

FIG. 9A. High resolution electron micrograph of neck cytoplasm of *Allogromia* after primary osmium fixation, without stabilization. Each vesicle (v) is bounded by a typical unit membrane. At (ip) note that the convolutions of the membranes give rise to invaginated profiles of membrane within the vesicles. The arrows indicate the presence around the dense bodies (db) of a membrane, which can only be detected as a single leaflet of the bilamellar leaflet usual for a unit membrane. Presumably the inner leaflet is fused to and masked by the dense material. There are no microtubules visible. Contrast with Figs. 9B, 10A, and 10B. Magnification approximately 147,000×.

FIG. 9B. Electron micrograph of neck cytoplasm after stabilization with a high

bodies are the principal elements in the population of particles which show bidirectional streaming in the living animal (Figs. 7–10). The dense bodies are also abundant in the cell body. Their composition is unknown, but they may conceivably be condensed remains of food organisms such as bacteria. At high magnification and resolution each dense body is seen to be enclosed by a membrane, the inner leaflet of which is so closely fused to the dense contrast contents that it is lost to vision (arrow, Fig. 9A). Although these particles are undoubtedly an important component of the population which shows active bidirectional movement, we have not yet obtained micrographs which display any specific association between them and the linear microtubules. The linear elements detected in our micrographs include only microtubules and elongate tubular profiles of smooth endoplasmic reticulum; we have not detected any microfilament array.

IV. Discussion

A. Organization of Reticulopodia

It seems clear from our sections of stabilized filopodia and reticulopodial strands that their basic organization differs from the earlier findings of Wohlfarth-Bottermann (1961) and of Lengsfeld (1969a,b,c). The entire pseudopod is surrounded by a single plasmalemma. This reopens the question of the apparent velocity gradients in this membrane, since particles attached to opposite sides of it are transported in opposite directions (Jahn and Rinaldi, 1959). Substantial rates of shear (in the order of 10 sec^{-1}) can be calculated as occurring in the membrane itself. One might postulate the presence of a surface mucus coat that is sheared, but such a coat has not been observed in our preparations (although seen by other workers in other networks; Hedley and Wakefield, 1969).

In this species of *Allogromia* no evidence has been found yet for either a "sol-gel" differentiation or the presence of a permanent "stereo-

concentration of magnesium chloride and double-fixation with glutaraldehyde and osmium (as described in Fig. 5). Note the abundance of longitudinal profiles of microtubules (mic) sweeping in a curved path through the neck region. Smooth membrane-bounded vesicles (v) are frequent, but have a noticeably less convoluted appearance than in Fig. 9A. Dense bodies (db) and mitochondria (mt) are present, as usual. Magnification approximately 106,600×.

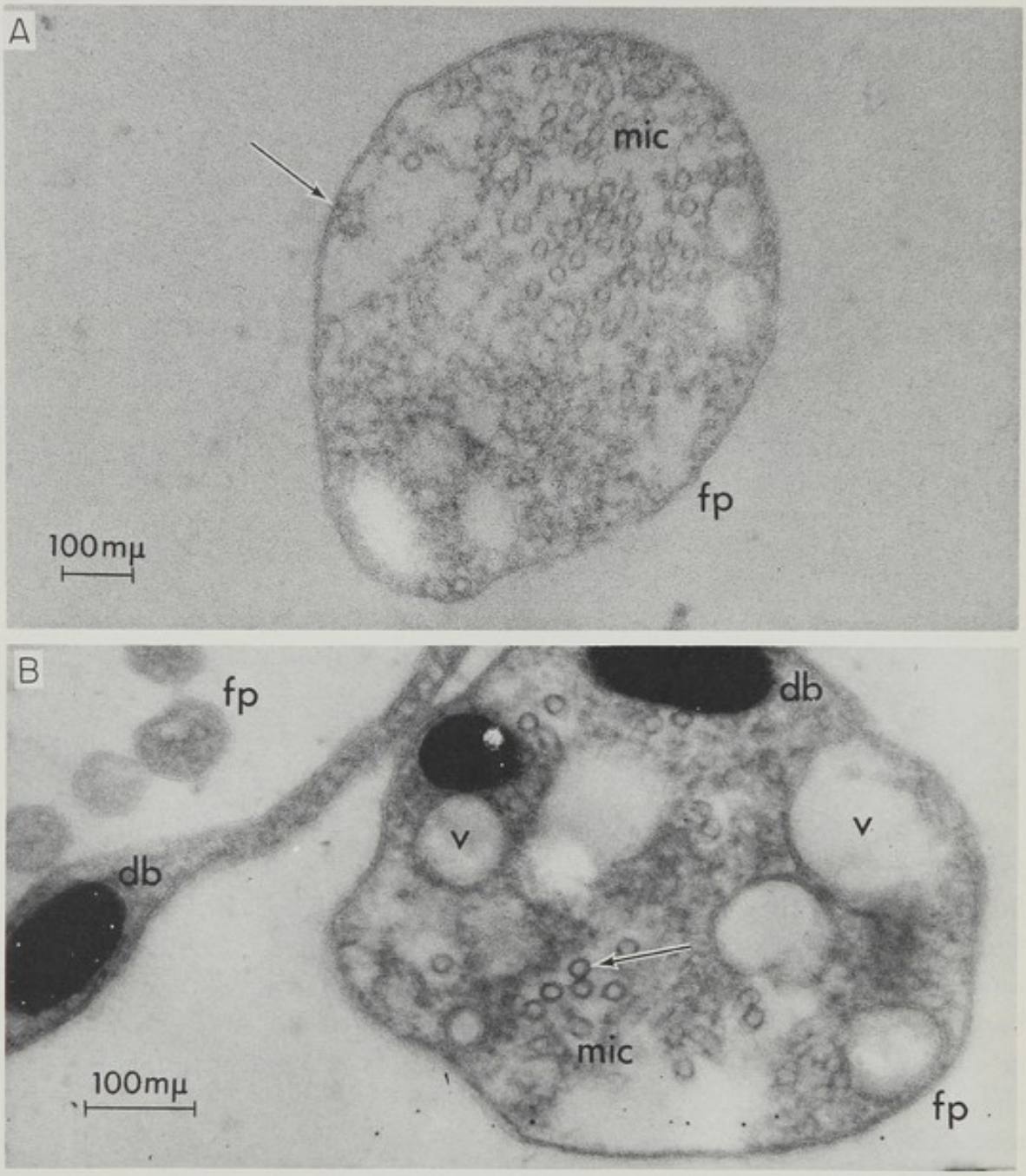

FIG. 10A. Transverse section through a stabilized filopodium, fixed with glutaraldehyde and osmium as described in Fig. 5. There is a complete plasmalemma (arrow) enclosing the filopodium (fp), and a localized group of microtubules (mic) some of which are cut very slightly off the true transverse plane. Magnification approximately 133,300×.

FIG. 10B. Transverse sections through stabilized filopodia (fp). Note the extremely small diameter of the filopods in the top left-hand corner, and the even thinner width of the pseudopodial element cut more longitudinally. A plasmalemma is clearly visible on all filopodia as a typical unit membrane. In the larger filopod, note the transverse sections of microtubules (mic) and the close apposition of two to form a pair (arrow). Dense bodies (db) and smooth membrane-bound vesicles (v) are also present. Magnification approximately 200,000×.

plasmic rod" comparable to the axonemes of heliozoans such as *Echinosphaerium* (Kitching, 1964; Tilney, 1967, 1968b; Tilney and Byers, 1969; Tilney and Porter, 1965; Tilney *et al.*, 1966; MacDonald and Kitching, 1967.) This finding confirms those of most recent authors. Adshead (1966) claimed that certain planktonic foraminifera undergo marked changes in the physical properties of their reticulopodial networks; for example, globigerinid pseudopodia supposedly develop thick axial cores and no longer anastomose. It would be interesting to find out whether these axial cores are microtubular, and if so whether microtubular lability has been lost.

B. Labile Microtubules in Pseudopod Extension and Retraction and in Cytoplasmic Streaming

It seems very likely that we have succeeded in demonstrating a class of highly labile microtubules in rapidly stabilized and fixed reticulopodia. The possible alternative view, that we might have *created* microtubules out of previously demonstrated microfilaments, seems unlikely both from what is known about the assembly of microtubules (Tilney, 1968a) and from the observed violent reactions of reticulopodia toward the conventional fixatives that lead to the finding of microfilaments. Porter (1966) has made the point that those microtubules which are more transient are less stable in fixation.

The finding of labile microtubules in the reticulopodial network does not immediately explain pseudopodial extension and retraction or streaming. It does, however, suggest a number of new and intriguing possibilities that are directly testable with present methods. Theoretical considerations are somewhat hampered by the unanswered question whether streaming is a consequence of the forces of pseudopod extension and retraction, or whether streaming is the "driven" process and the formation of pseudopods is a by-product. However, from a behavioral standpoint the former point of view is more attractive because several authors have stressed the delicate response of foraminiferan filopodia to food (Leidy, 1879; Doflein, 1916; Jepps, 1942).

Each of the following possibilities has its obvious predictions at the ultrastructural level.

1. Microtubules may be pushed out of, or reeled into, the cell body by active shearing or sliding among the microtubules themselves. Such an idea was advanced before the discovery of microtubules by Noland (1957) and later elaborated and applied to *Allogromia laticollaris* by

Jahn and Rinaldi (1959). More recently, observations of cross bridges between adjacent spindle microtubules (McIntosh, 1968; Hepler and Jackson, 1968; Wilson, 1969; Brinkley *et al.*, 1969) have led to the formulation of a detailed model of how such a process might account for movements in the mitotic spindle (McIntosh *et al.*, 1969). The advantage of this active shearing mechanism is that it can be applied to pseudopod extension and retraction, streaming, and rapid withdrawal. The only difficulty is that it does not account for the observed velocities of particle movements without a number of additional assumptions (Allen, 1964). Testing the model requires a detailed "wiring diagram" of microtubules in an extended network; it also requires that the microtubules be contiguous or attached wherever the force is applied.

2. A "multiple contraction sites" model of reticulopodial streaming also predicts testable ultrastructural consequences. The model of Allen (1964) proposed contractile events at pseudopod tips and at attachment points, so that the entire network would not be dependent upon forces generated either in the body alone or in all parts of the network, but would be kept in motion by independent "booster motors."

The intriguing effects of temperature on axonemal microtubules in *Echinosphaerium* (Tilney, 1967) suggest one way in which a microtubule could shorten and apply a force. Tilney found that low temperature transforms microtubules from one state to another, involving a significant increase in diameter, a change in the angle of the rows of subunits, and presumably also a significant shortening. Such a change of conformation within a microtubule might also occur under physiological conditions and result in the generation of a force. This possibility is attractive because it would be consistent with the observed velocities of particles approaching and leaving filopodial tips. Although nothing is known so far about the proteins responsible for motility in foraminifera, we might expect to find indications of microtubular cross-bridges and myosin-like, or actin-like molecules in regions where contraction is taking place.

3. It is also possible that microtubular assembly and disassembly could be sufficiently rapid to account for pseudopod extension and withdrawal and for cytoplasmic streaming. Such a model would also have to be tested by working out the wiring diagram of microtubules; one prediction would be free ends of microtubules. That the network is capable of rapid microtubular disassembly is shown by the break-up of filopodia on interspecific contact (Allen, 1964). Beading on stimulation or injury also seems to be the effect of surface tension on a filopod suddenly deprived of its gel structure.

Foraminifera that are establishing or retracting extensive networks

transport "droplets" of cytoplasm, derived from retracting portions, toward regions of network growth. Such droplets may be transported at the tips of filopodia (the "bouton terminale" of Le Calvez, 1938) or along extending or retracting pseudopodia. The involvement of droplets in the retraction of old networks and the building of new ones has been referred to as the "fiber $\rightleftharpoons$ droplet transition" (Allen, 1964). It may represent nothing more or less than a visible manifestation of rapid assembly and disassembly of microtubules.

At the present time we do not have any reliable data on the rates of assembly and disassembly of microtubules in *Allogromia*. Consequently, it is not possible to assess whether this process alone could account for pseudopod extension and retraction or for cytoplasmic streaming.

C. Control of Body Form and the Differentiation of Neck Cytoplasm

The existence of monopolar, dipolar, and multipolar cells suggests that some mechanism exists for making and controlling the number of oral apertures. The key to understanding this process may lie in the differentiation of "neck cytoplasm," which is ultrastructurally different from "body cytoplasm." As shown in Table I, the two types differ in their respective contents of organelles and linear elements of the ground cytoplasm. The details of oral aperture formation require further detailed study from a developmental standpoint.

D. Application of These New Methods to Other Cells

The methods outlined in this paper are applicable to combined light and electron microscopic studies of many other cells. In this laboratory, they have been applied so far with success to tissue cultures of nerve cells, *Haemanthus* endosperm cells, crane-fly spermatocytes, and fresh water protists.

It is quite possible that labile microtubules are more generally distributed in cells than is now recognized, especially in highly motile cells. Rapid presentation of a stabilizing agent and subsequent rapid fixation may prove very generally useful. In addition, detailed studies of the various phases of behavioral responses by the reticulopodial net-

work of *Allogromia* should reveal the significance of "fast" labile microtubules for cell movement.

V. Summary

Observation and microperfusion chambers have been used for a study of the reticulopodial network of the marine foraminiferan *Allogromia* sp. (strain N. F. Lee), employing closely correlated light and electron microscopy. Conventional fixatives were found to produce severe damage and to change the form of the network. This could be prevented by treating the animal before fixation with a stabilizing solution, consisting of 38% magnesium chloride solution in Instant Ocean, which produces virtually instantaneous cessation of movement and preservation of lifelike form. Subsequent treatment with conventional fixatives then produced no visible damage. A lower concentration of $MgCl_2$, 7.5% in Instant Ocean, produced a patterned sequence of behavioral response in the filopods, followed by stabilization and cessation of movement; this permitted some experimental control over the state of the reticulopodial network. The effects of treatment with both concentrations of $MgCl_2$ in Instant Ocean were fully reversible. Although no microtubules were detected after primary fixation with glutaraldehyde and osmium tetroxide, microtubules are abundant in reticulopodia when fixation is preceded by stabilization. The possible significance of highly labile microtubules is discussed. The methods developed are applicable to a wide variety of single cell systems for correlated light and electron microscopy.

Acknowledgments

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REFERENCES

Adshead, P. C. (1966). *Amer. Ass. Petrol. Geol., Soc. Econ. Paleontol. Mineral., 41st Annu. Meet., Long Beach, Calif., Program Abstr.* pp. 16–17.

Allen, R. D. (1964). *In* "Primitive Motile Systems in Cell Biology" (R. D. Allen and N. Kamiya, eds.), pp. 407–432. Academic Press, New York.

Allen, R. D. (1968). *Symp. Soc. Exp. Biol.* **22**: 151–168.
Allen, R. D., and Drum, R. W. (1969). *Biol. Bull.* **137**: 391.
Allen, R. D., David, G. B., and Nomarski, G. (1969). *Z. Wiss. Mikrosk.* **69**: 193-221.
Angell, R. W. (1967). *J. Protozool.* **14**(4): 566–574.
Arnold, Z. M. (1953). *Contrib. Cush. Found. Foram. Res.* **4**: 15–21.
Bajer, A. (1968). *Symp. Soc. Exp. Biol.* **22**: 285–310.
Bender, C., Flynn, J., Rollins, E., and McGee-Russell, S. M. (1970). In preparation.
Brinkley, B. R., Cartwright, J., Jr., and Murphy, P. (1969). *J. Cell Biol.* **43**: 169a–170a; *Amer. Soc. Cell Biol. Abstr.* **408.**
Doflein, F. (1916). *Zool. Jahrb. Abt. Anat. Ontog. Tiere* **39**: 335–384.
Hedley, R. H., and Wakefield, J. St. J. (1969). *Bull. Brit. Mus. (Natur. Hist.)* **18**: 69–89.
Hedley, R. H., Parry, D. M., and Wakefield, J. St. J. (1967). *J. Roy. Microsc. Soc.* **87**: 445–456.
Heilbrunn, L. V. (1952). "An Outline of General Physiology." Saunders, Philadelphia, Pennsylvania.
Hepler, P. K., and Jackson, W. T. (1968). *J. Cell Biol.* **38**: 437–446.
Jahn, T. L., and Rinaldi, R. A. (1959). *Biol. Bull.* **117**: 100–118.
Jepps, M. W. (1942). *J. Mar. Biol. Ass. U.K.* **25**: 607–666.
Kamiya, N. (1968). *Symp. Soc. Exp. Biol.* **22**: 199–214.
Kamiya, N., and Nakajima, H. (1955). *Jap. J. Bot.* **15**(1): 49–55.
Kitching, J. A. (1964). *In* "Primitive Motile Systems in Cell Biology" (R. D. Allen and N. Kamiya, eds.), pp. 445–456. Academic Press, New York.
Le Calvez, J. (1938). *Arch. Zool. Exp. Gen.* **80**: 163–333.
Leidy, J. (1879). *Rep. U.S. Geol. Surv.* **12**: 1–324.
Lengsfeld, A. M. (1969a). *Helgolaender Wiss. Meeresunters.* **19**: 230–261.
Lengsfeld, A. M. (1969b). *Helgolaender Wiss. Meeresunters.* **19**: 262–283.
Lengsfeld, A. M. (1969c). *Helgolaender Wiss. Meeresunters.* **19**: 385–400.
Luft, J. H. (1961). *J. Biophys. Biochem. Cytol.* **9**: 409–414.
MacDonald, A. C., and Kitching, J. A. (1967). *Nature (London)* **215**: 99–100.
McGee-Russell, S. M., and Allen, R. D. (1969). *J. Cell Biol.* **169**: 88a, 167a.
McIntosh, J. R. (1968). *J. Cell Biol.* **39**: 89a.
McIntosh, J. R., Hepler, P. K., and Van Wie, D. G. (1969). *Nature (London)* **224**: 659–663.
Molé-Bajer, J., and Bajer, A. (1968). *Cellule* **67**: 257–265.
Nagai, R., and Kamiya, N. (1966). *Proc. Jap. Acad.* **42**: 934.
Nagai, R., and Rebhun, L. I. (1966). *J. Ultrastruct. Res.* **14**: 571–589.
Noland, L. E. (1957). *J. Protozool.* **4**: 1–6.
Padawer, J. (1968). *J. Roy. Micros. Soc.* **88**: 305–349.
Palay, S. L., McGee-Russell, S. M., Gordon, S., and Grillo, M. A. (1962). *J. Cell Biol.* **12**: 385–410.
Porter, K. R. (1966). *In* "Principles of Biomolecular Organization," Ciba Found. Symp., pp. 308–345. Little, Brown, Boston, Massachusetts.
Rinaldi, R. A., and Jahn, T. L. (1964). *Protoplasma* **58**: 369–390.
Sandon, H. (1934). *Nature (London)* **133**: 761–762.
Sandon, H. (1944). *Nature (London)* **154**: 830–831.
Sandon, H. (1961). *Progr. Protozool., Proc. 1st Int. Conf. Protozool., Prague,* pp. 166–169.
Tilney, L. G. (1967). *J. Cell Biol.* **34**: 327–343.
Tilney, L. G. (1968a). *Develop. Biol. Suppl.* **2**: 63–102.

Tilney, L. G. (1968b). *J. Cell Sci.* **3**: 549–562.
Tilney, L. G., and Byers, B. (1969). *J. Cell Biol.* **43**: 148–165.
Tilney, L., and Porter, K. R. (1965). *Protoplasma* **60**: 317–334.
Tilney, L. G., Hiramoto, Y., and Marsland, D. (1966). *J. Cell Biol.* **29**: 77–95.
Wilska, A. (1953). *Nature (London)* **171**: 353, 698.
Wilska, A. (1954). *Mikroskopie* **9**: 1–80.
Wilson, H. J. (1969). *J. Cell Biol.* **40**: 854–859.
Wohlfarth-Bottermann, K. E. (1961). *Protoplasma* **54**(1): 1–26.

CHROMOSOME ALTERATIONS IN HUMAN CARCINOGENESIS*

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I. Introduction

Chromosome alterations as a cause of malignant neoplasms was postulated more than 50 years ago by Boveri (1914) and has been debated ever since then. Although the causes of cancer still remain obscure, we know that many types of cancer arise from a single cell that undergoes one or more permanent hereditary changes and thereafter multiplies, giving rise to billions of similarly altered cells. Because cancer or neoplasia is a disease which can exist only in multicellular organisms, other conditions such as failure of the immunological defenses of the organism may be required for the development of cancer. Nevertheless, the fundamental event in many cancers is the irreversible alteration of that one initial cell.

The simplest explanation for a hereditary cellular change is that there is some change in deoxyribonucleic acid (DNA), inasmuch as this is

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the hereditary material, preserved and replicated in the chromosomes of each cell in a constant and precise manner to guarantee the hereditary continuum. Mutations, which interrupt this continuum, may be of different kinds; they can originate in changes in the chromosomal DNA itself, they can involve the chromosomal proteins, or they can result from irregularities in chromosome movement during the mechanically active phases of division.

Morphologically, gross alterations of the chromosomal material are usually detectable as changes in chromosome number at the karyotype level, or as structural changes at the level of individual chromosomes. Such abnormalities will be shown in this article to be present in human malignant neoplasms. Furthermore, because new insights have become available into the ultrastructure of human chromosomes as studied by electron microscopy, alterations of chromosomal ultrastructure have been demonstrated in human cancer cell lines. Additionally, a new quantitative technique of electron microscopy has made it possible to quantify these ultrastructural changes and to propose a model of the DNA arrangement within the chromosomes. This DNA arrangement (or DNA packing ratio) appears to be altered in chromosomes from cancer cells.

II. Alterations of Metaphase Chromosomes in Human Malignant Cells

A. Alterations of the Karyotype

1. Human Congenital Diseases and Cancer

The first step in learning something about chromosomes and cancer is to examine the relationship with congenital diseases.

The most common human chromosomal anomaly is Down's syndrome (mongolism; frequency 1:600 in newborn babies); in this condition, which is compatible with life, 96% of all patients have 47 chromosomes in their somatic cells, the extra one being a 21–22 chromosome. The increasing incidence of Down's syndrome among offspring of mothers more than 35 years old (frequency 1:100 in mothers over 40) suggests that the extra chromosome arises by nondisjunction during female gametogenesis. One of the causes of nondisjunction might be the attachment of the involved acrocentric chromosome 21–22 to a (virus-induced?) persistent nucleolus during meiosis in the aging human oocyte (Evans, 1967).

Excessive maternal age not only favors the risk of mongolism but

also of leukemia (Stark and Mantel, 1966). It is not surprising, therefore, that leukemia and mongolism occur together quite often. In statistical surveys, acute leukemia was found to be associated with mongolism from three times (Krivit and Good, 1957) to 20 times (Stewart *et al.*, 1958) more frequently than expected. Chromosome analysis of leukemic cells from these mongoloid children usually does not show any other abnormality except the extra chromosome 21–22 (Fig. 1; Lampert, 1967). However, in some cases additional chromosome anomalies have

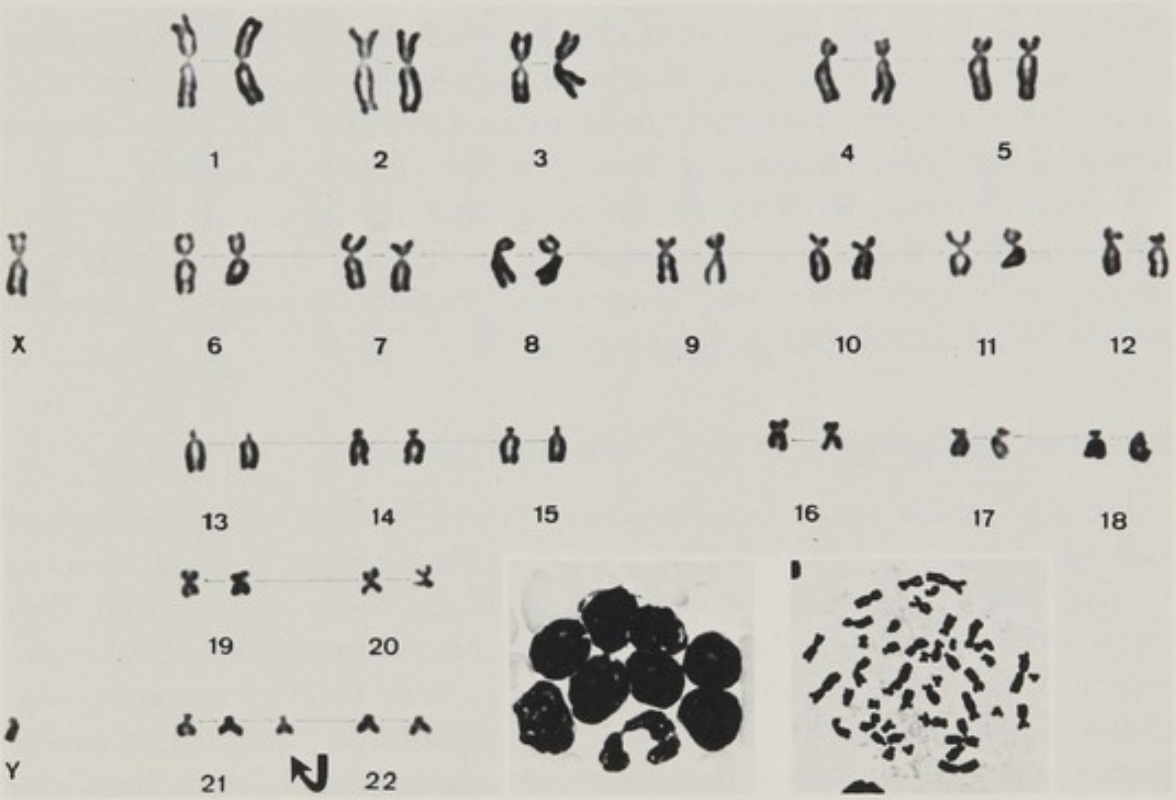


FIG. 1. Karyotype of a 6-year-old boy with mongolism and acute lymphoblastic leukemia. Metaphase and interphase of leukemic cells from the bone marrow are also shown. No chromosomal aberration is seen except the trisomy 21.

been found (Conen and Erkman, 1966). Although the common factor which operates to favor simultaneous occurrence of leukemia and mongolism is not known, it seems likely that an organism containing cells with an altered and instabile karyotype may be prone to additional defects, e.g., immunological deficiencies which could make that person prone to cancer.

In looking further for inborn genetic factors in the etiology of cancer, an investigator comes upon three diseases caused by autosomal recessive genes and involving vascular, hematological, or neurological systems, which are accompanied by a predisposition for leukemia and other lymphoreticular malignancies; these are Bloom's syndrome, Fanconi's anemia

and ataxia telangiectasia. Reports by German *et al.* (1965) on Bloom's syndrome, Schroeder *et al.* (1964) on Fanconi's anemia and Hecht *et al.* (1966) on ataxia telangiectasia demonstrate that the somatic cells in these three conditions have in common an unusual chromosome fragility. There is a high frequency of chromosome breakage producing chromatid gaps, isochromatid breaks, abnormal acrocentric chromosomes, acentric fragments, and quadriradial figures. Furthermore ataxia telangiectasia is characterized by impaired development of lymphoid tissue (Peterson *et al.*, 1966) and impaired responsiveness of lymphocytes to phytohemagglutinin (Hecht *et al.*, 1966), resulting in severe immunological deficiency. It is interesting that in siblings having ataxia telangiectasia, acute lymphoblastic leukemia develops at the usual age peaks known for childhood leukemia, namely 3 and 14 years (Lampert, 1969a).

In summarizing the findings on congenital diseases and cancer, we can state that there is a prevalence of malignancy in individuals possessing cells with increased chromosome fragility, especially if accompanied by immunological incompetence.

2. *Aneuploidy in Human Cancer Cell Populations*

Usually cancer arises in an organism which, except for the malignant cell population, is composed of somatic cells that are perfectly normal in their karyotypes. Consequently, it is all the more striking that high degrees of aneuploidy, involving few or many supernumerary chromosomes, occur in the genomes of cancer cells. At first sight these numerical changes seem to be the result of a very disorderly process, but earlier pathocytologists proposed that there are chromosomal "stem lines" for different cancer cell populations and that these arise by systematic evolution of the karyotype (Makino, 1957). An established cancer cell population may be characterized by a modal chromosome number, which represents the majority of the cells, which is relatively stable over periods of months or years, and which can be observed unaltered after prolonged cultivation either *in vitro* or *in vivo*, e.g., in leukemic cell populations during successive relapses or in metastases of solid tumors. It seems that the altered karyotype, once it has arisen and proved to be of selective advantage, is transmitted essentially unchanged to the daughter cells in the growing cancer cell population. Generally speaking, no two karyotypes are identical in human cancer or acute leukemia, and no typical chromosome group has been found to be involved (Sandberg *et al.*, 1968a); however, chronic granulocytic leukemia is an exception which will be reserved for later discussion in this article.

Among human cancer conditions, the acute leukemias offer some ad-

vantages for chromosome studies: For example, direct bone marrow techniques allow chromosome preparations without resort to culture. Bone marrow samples can be obtained during either relapse or remission of the disease, and consequently leukemic cells can be compared directly with the normal diploid marrow cells. Furthermore, acute leukemia during childhood is not only the most frequent malignant disease (half of all cancer deaths in children are due to leukemia) but also is the

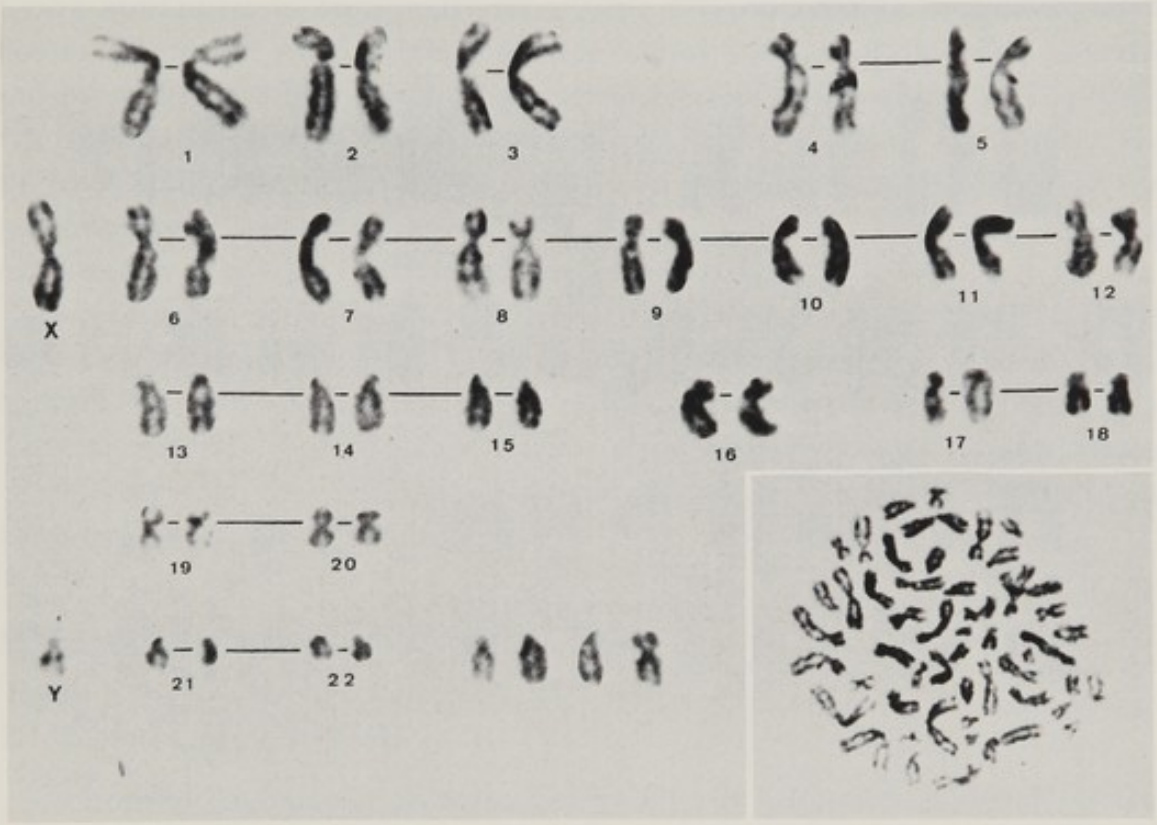


FIG. 2. Karyotype and metaphase of a leukemic cell from the bone marrow of a 3½-year-old boy with acute lymphoblastic leukemia. The modal chromosome number is 50; note the general fuzziness of chromosomes.

one disease which nowadays kills the most children in North America and Europe (4 out of 100,000 per year). In contrast to adult leukemias, lymphoblastic cell types are found in 80% of childhood cases.

An example of direct chromosome analysis in a patient with acute lymphoblastic leukemia is presented in Fig. 2; there is a strikingly hyperdiploid chromosome number. This aneuploidy in the bone marrow cells disappears during therapeutically induced remission, when the bone marrow regenerates its normal white and red cell precursors. However, during relapse the same chromosome aneuploidy reappears as the marrow becomes densely populated again with leukemic cells. Apparently the chromosomal stem line of the leukemic cell population is not changed by chemotherapy. Relapse occurs by remultiplication of a few

leukemic cells which survive the onslought of intensive chemotherapy in some protected spaces of the body. The appearance of aneuploid metaphases in a previously diploid marrow is also of practical clinical value because it foreshadows imminent relapse and calls for a change of therapy. Whether the chromosome constitution of the malignant cells in acute leukemia is also of prognostic value in that aneuploid cells are more sensitive to chemotherapy than diploid cells remains to be seen (Lampert, 1969b).

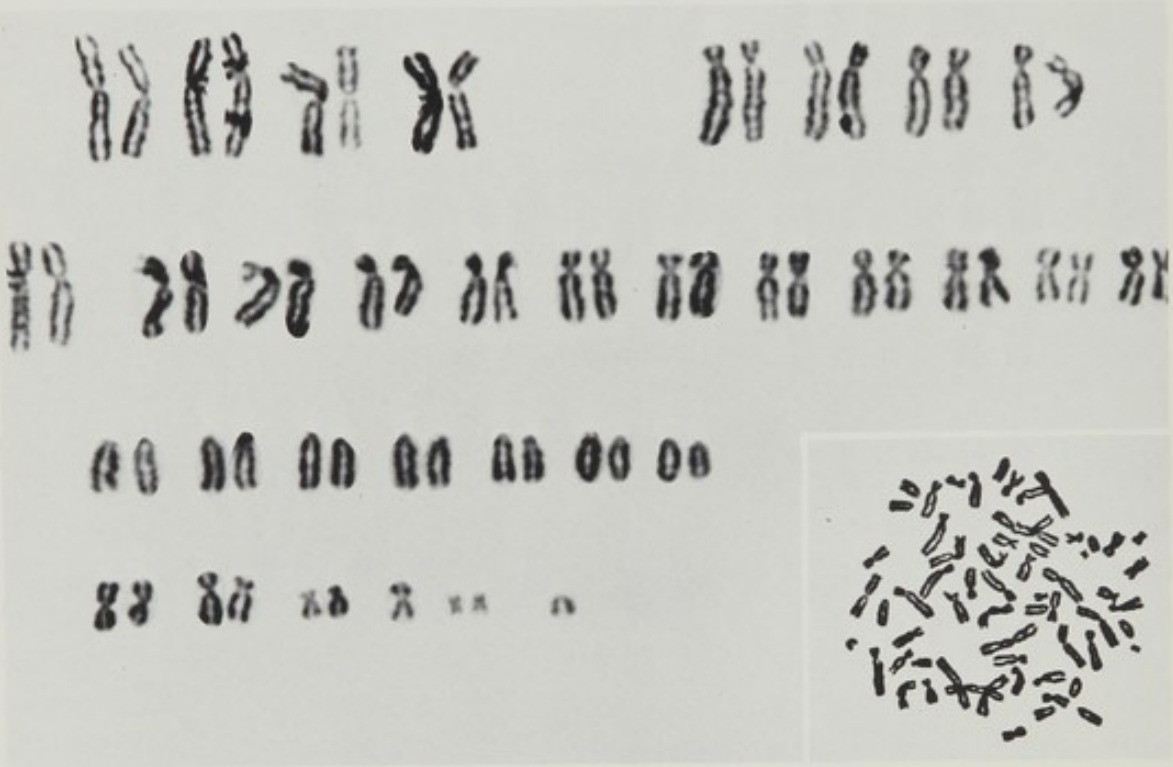


FIG. 3. Karyotype and metaphase of a human ovarian carcinoma (GW-127) which was serially transplanted to hamster cheek pouches. Extreme aneuploidy with 64 chromosomes is seen.

In the most comprehensive study (219 cases) of chromosomes in the marrow cells of acute leukemia (Sandberg *et al.*, 1968b), several important features were found; aneuploidy was present only in 50% of the cases, i.e., about 50% of the patients with acute leukemia had a completely normal appearing diploid karyotype in their malignant cells. Even in the aneuploid cases, no consistent or characteristic chromosomal change could be identified. However, hyperdiploidy was more characteristic for acute lymphoblastic leukemia (ALL) whereas acute granulocytic leukemia (AGL) was accompanied by either hypo- or hyperdiploidy. In 40% of the aneuploid cases of acute leukemia, abnormal (marker) chromosomes were present in the leukemic cells. These marker chromosomes ranged from minute objects to submetacentric chro-

mosomes much larger than a No. 1 chromosome. By contrast with solid tumors, small or large acrocentric markers were not common in acute leukemia.

In clinical patients, either primary or metastatic solid tumors differ somewhat from acute leukemia in that they often have aneuploid modes in the triploid range, i.e., 62–85 chromosomes (Yamada *et al.*, 1966). These cancer cell karyotypes, especially when they are propagated in living animal hosts, frequently have little resemblance to human chromosomes (Lampert *et al.*, 1968). Figure 3 shows a karyotype with 64 chromosomes from a human ovarian carcinoma cell line which was serially transplanted *in vivo* to hamster cheek pouches. Cells of this tumor are highly "malignant"; they grow rapidly in the heterologous host, invade surrounding tissue, and metastasize into a variety of organs, breaking all barriers of species, individual, organ or tissue specificity.

In summary, aneuploidy with many supernumerary chromosomes that lack any definite pattern is a characteristic feature of human solid tumors. In acute leukemia, aneuploidy occurs only in 50% of the cases. The irregularity and protean nature of these karyotypic changes makes it unlikely that they are direct causes of neoplasia, but probably represent secondary phenomena.

B. Internal Alterations of Chromosomes

1. *Marker Chromosomes*

Abnormal chromosomes occur frequently in human cancer cells; however, the number of abnormal chromosomes in each cancer cell population is usually limited to 2 or 3. If these abnormal chromosomes have a characteristic appearance and are found in all or most of the examined metaphases they are called marker chromosomes. Continuous presence of the same marker chromosome in many or all tumor cell metaphases strongly suggests that these tumors arise from a single cell in which a structural rearrangement of the chromosomes has occurred (Atkin and Baker, 1966). Perhaps the most convincing argument for a causal relation between a specific chromosome abnormality and a form of human malignancy concerns a marker chromosome found in chronic granulocytic leukemia (CGL).

a. Philadelphia (Ph^1) Chromosome in Chronic Granulocytic Leukemia. Chronic granulocytic (or myelocytic) leukemia is a neoplastic blood disease of adults, characterized by an increase in white blood cell count (over 50,000 per mm^3), marked enlargement of the spleen,

and a fatal course within 3 to 4 years. The averaged alkaline phosphatase activity of neutrophilic white blood cells is markedly reduced in this disease due to absence of the enzyme from the malignant granulocytes. In 1960, Nowell and Hungerford discovered that the malignant cells of patients with CGL contain a chromosomal aberration that evidently arises as a deletion of part of the long arm of one of the four small acrocentric chromosomes (either 21 or 22; see Fig. 4). According to DNA measurements by Rudkin *et al.* (1964), the partial deletion which produces this abnormal chromosome amounts to about 40% of a normal

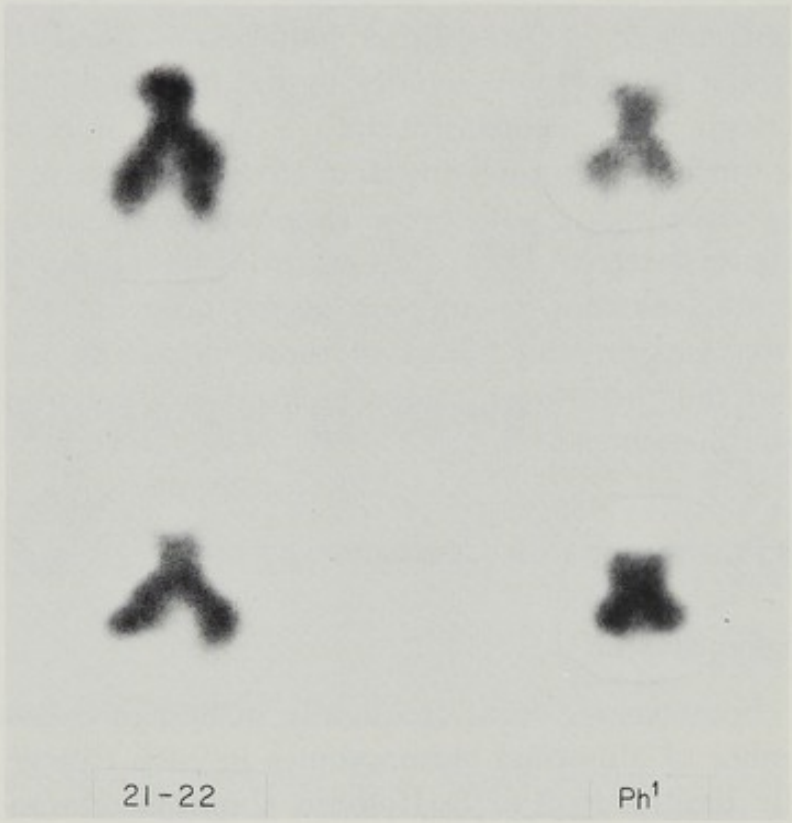


FIG. 4. A 21–22 chromosome and a Philadelphia chromosome (Ph^1) from two bone marrow cells of a patient with chronic myelocytic leukemia. The Philadelphia chromosome is thought to arise from a chromosome 21 by a long arm deletion.

chromosome 21–22. The result is the so-called Philadelphia (Ph^1) marker chromosome, which appears to be specific for CGL or closely related myeloproliferative disorders.

In a study of 179 consecutive patients with the clinical and laboratory findings of CGL, 158 (or 88%) were found to have Ph^1 (Whang *et al.*, 1968). It also appears that the deletion responsible for Ph^1 is most likely a postzygotic event; in five of six cases of monozygotic twins, only the leukemic twin had the Ph^1 chromosome (Jacobs *et al.*, 1966; Kosenow and Pfeiffer, 1969). Ph^1 has also been found to be present before the clinical (or symptomatic) phase of the leukemic process

(Kemp *et al.*, 1964). Although Ph[1] occurs only in hematopoietic cells and is not detectable in other somatic cells of the patient such as skin fibroblasts, it is not confined to granulocytic cell lines but is present in the erythrocytic (Clein and Flemans, 1966) and megakaryocytic (Tough *et al.*, 1963) cell lines of the bone marrow. One can postulate, therefore, that this chromosome originates in an ancestral stem cell common to all three cell lines; this lends further support for the common stem cell hypothesis as applied to bone marrow precursors (Whang *et al.*, 1963). Detection of the Ph[1] chromosome is also of prognostic value, because it is always present in bone marrow and its reappearance in blood during remission foreshadows a new relapse.

During the chronic phase of CGL, Ph[1] is the only detectable chromosome change in the majority of cases, whereas in the acute terminal stages Ph[1] is often associated with other chromosome abnormalities that vary considerably from patient to patient (Fitzgerald, 1966; Castro-Sierra *et al.*, 1967). Often a clonal evolution can be observed in these cell populations, beginning with Ph[1] and proceeding either to successive acquisitions of supernumerary chromosomes or to successive loss of chromosomes especially in groups 21–22, 17–18, and 6-X-12 (Grouchy *et al.*, 1966).

In summary, the Ph[1] chromosome is a unique phenomenon among the leukemias and has a strong claim to be associated with the etiology of chronic myelocytic leukemia; however, a causal role has not yet been established.

b. Large Acrocentric Chromosomes in Solid Tumors. Cells of solid tumors such as carcinomas of the uterus, ovary, breast, testis, stomach, colon, lung, and various other epithelial or nonepithelial tumors very often possess marker chromosomes (Makino *et al.*, 1959, 1964; Ishihara *et al.*, 1961, 1963; Grouchy *et al.*, 1963a; Atkin and Baker, 1966; Miles, 1967; Goh, 1968; Rigby, 1968). Usually these markers are large acrocentrics with sizes comparable to an A, B, or C chromosome; however, in some cases the markers are ring or dicentric chromosomes. The protruding long arms of a large marker chromosome can be so prominent that they are detectable even in histological sections of tumors (Brandão and Atkin, 1968). Although in some tumors, such as Burkitt's lymphoma, a large acrocentric marker chromosome is present in most of the cases (Jacobs *et al.*, 1963), specificity of a recognizable marker for a certain malignant disease has been found only in chronic granulocytic leukemia. Apparently similar markers, such as these large acrocentric chromosomes, are often seen in tumors of very different origin. Why the abnormal chromosomes tend to prefer having their kinetochores in acrocentric

positions is not known. Perhaps large acrocentric chromosomes are better able to survive in these more or less rapidly replicating cancer cell populations and consequently are produced by selection pressures.

A large marker chromosome of this type from Burkitt's lymphoma has been characterized precisely by quantitative electron microscopy (Lampert *et al.*, 1969). With this procedure, it has been possible to infer which chromosome in the normal karyotype probably generated the marker by a structural rearrangement (see Section C). However, the primary reason why certain chromosomes like the large chromosomes No. 1, 2, and 3 are consistently involved in the structural changes of different tumors still remains elusive. The practical application of the search for marker chromosomes is that they can help in deciding whether a given tumor is of single- or multicell origin (Atkin, 1967). All the available data so far support the view that human tumors usually arise from a single cell.

In summarizing data about marker chromosomes, it can be stated that, except for the Philadelphia chromosome in CGL, there is no specific chromosome abnormality associated with a human malignant disease. Production of marker chromosomes is probably a secondary event following the still unknown alterations that change a normal cell into a cancer cell. However, persistence of marker chromosomes in malignant cell populations points to a single-cell origin for most human tumors.

2. *Other Structural Changes of Tumor Cell Chromosomes*

It is a common experience for those who do cytogenetic analysis of normal and malignant cells to find that chromosomes derived from malignant cells *in vivo* not only spread poorly on the microscope slide but also tend to stain lightly or unevenly, thus giving poor contrast in the light microscope. Nevertheless, it can be argued that no two chromosome preparations are alike and that chromosome spiralization is easily influenced by many external factors during the processes of cultivation and preparation. If one could prepare normal and tumor cell chromosomes from the same cell population without resort to culture or exposure to colchicine, the different appearance of tumor cell chromosomes might be established more precisely. This, in fact, is possible by preparing bone marrow specimens from children with acute lymphoblastic leukemia (ALL), who in the up and down course of their disease change from partial remission to early relapse. In these patients, the number of leukemic blast cells in the marrow does not exceed 50%, while the remaining cells are normal (including both red and white blood cell precursors). For example, direct chromosome preparations from the bone

marrow of one patient revealed an aneuploid malignant stem line of 59 and a diploid line of 46 chromosomes. These metaphases, some derived from normal bone marrow cells and others from leukemic blast cells, could be found on the same slide; the difference in appearance and staining characteristics was quite striking. Whereas the metaphase with 46 chromosomes and a diploid karyotype consisted of thin, well-delineated and well-defined chromatids, the aneuploid metaphase was

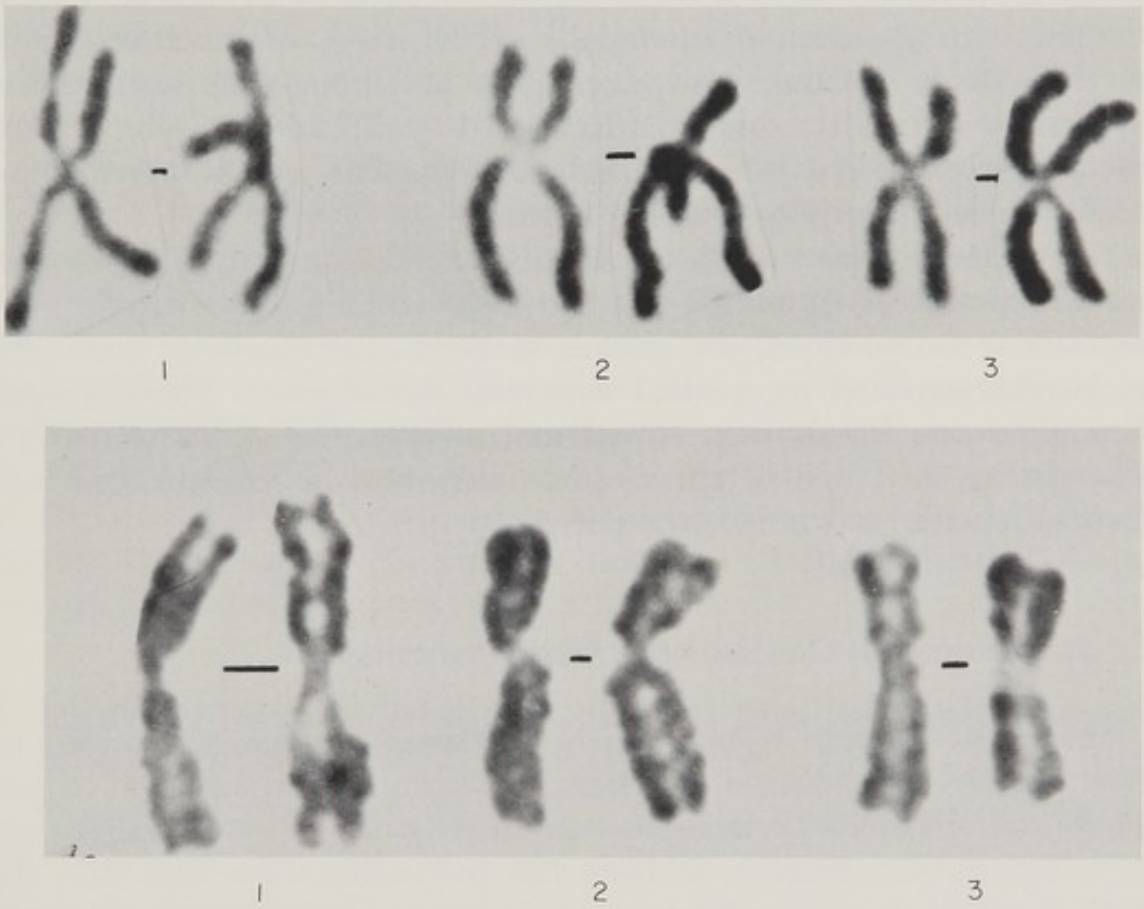


FIG. 5. Chromosomes No. 1, 2, and 3 from a 2½-year-old boy with acute lymphoblastic leukemia in partial remission. Chromosomes in the top row are from a normal cell with 46 chromosomes, and in the bottom row from a leukemic cell with 59 chromosomes. Both were prepared identically from the same bone marrow aspirate. Note the characteristic differences in chromosome appearance (described in the text).

characterized by fuzzy, ill-defined, lightly or irregularly stained, and poorly separated chromatids. In Fig. 5, the group A chromosomes from a diploid metaphase and from an aneuploid metaphase containing 59 chromosomes have been compared side by side. Both metaphases were derived from the same direct bone marrow preparation of a child with ALL in partial relapse; the irregularity and poor separation of the leukemic chromatids is quite marked.

This difference in the appearance of normal and leukemic chromo-

somes, which is a frequent finding in human acute leukemia, has been reported previously by Sandberg (1966) and by Lampert (1968). It seems likely that these cancer cell metaphases express a common abnormality. Could it be caused by increased "stickiness" of the chromosomes or the chromatid material, a phenomenon which was observed even with inadequate preparative techniques for light microscopy more than 20 years ago (Koller, 1947)? What can be seen with the light microscope with a maximum resolution of 1700 Å should be readily clarified with the electron microscope, which has a maximum resolution of about 5 Å. Although the elucidation of chromosome substructure by electron microscopy has been hampered by difficulties in preparation, these obstacles have recently been overcome; an era of chromosome analysis at the Ångström level has begun.

In concluding this section on alterations of cancer cell chromosomes, we can state that in addition to the more striking abnormalities such as marker chromosomes, the chromosomes of cancer cells *in vivo* seem to be characterized by general structural disturbances. These general alterations can barely be resolved and analyzed by light microscopy. Elucidating this type of chromosome alteration at the ultrastructural level will be the task of electron microscopy.

C. Alteration of Chromosome Ultrastructure

Successful electron microscopy of chromosomes depends largely on how well the difficulties of cytological preparation are mastered. A precondition for examining metaphase chromosomes by electron microscopy is a high mitotic index of at least 10%, preferably more than 50%; this high a yield of mitotic figures can be achieved by arresting rapidly growing cells at metaphase with colchicine or vinblastine in doses of about 0.02 μg per milliliter of culture medium for 10 hours or more. Also, in monolayer cultures, dividing cells can be shaken off the bottle wall, thus synchronizing the cell population in mitosis. All these procedures give variable success, depending on the cell lines cultured or stimulated to grow *in vitro*.

All our data regarding the ultrastructure of chromosomes from human tumor cells have been derived from *in vitro* cultures. However, similar studies of direct chromosome preparations from *in vivo* tumor tissue or leukemic bone marrow are planned, in order to examine the fuzzy, agglutinated, disordered tumor chromosomes at an ultrastructural level. The best method of preparing isolated unsectioned chromosomes as whole mounts for electron microscopy is a combination of surface

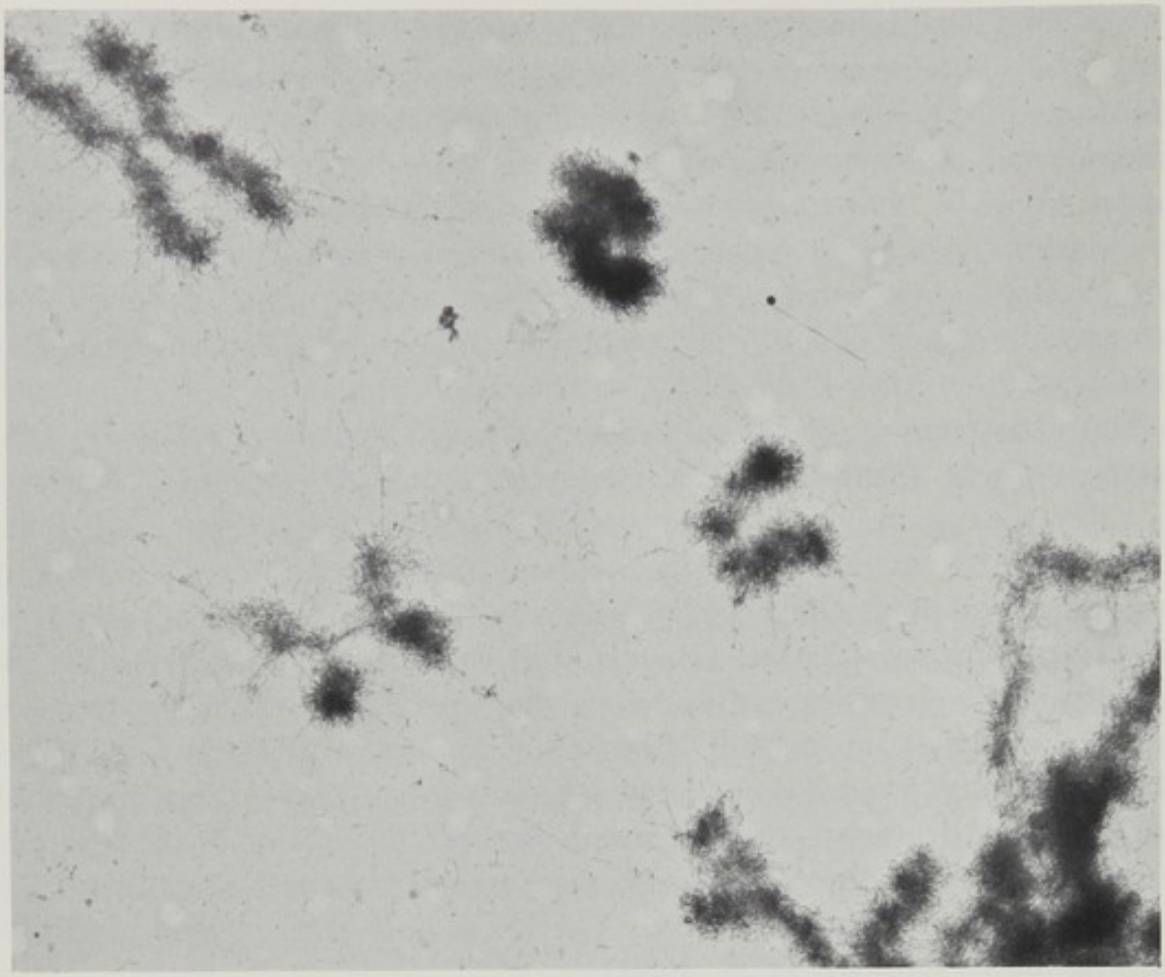
spreading (Kleinschmidt *et al.*, 1959) and critical point drying (Anderson, 1951); this procedure was first successfully applied to human chromosomes by DuPraw (1966). Critical point drying preserves the three-dimensional structure by preventing artifacts of air drying caused by surface tension. Preparations spread and dried by this method clearly demonstrate the fiber substructure of chromosomes: i.e., chromosomes from animal and human cells both at metaphase and interphase are composed entirely of fibers 200 to 500 Å in diameter (Ris and Chandler, 1963; DuPraw, 1965; Gall, 1966).

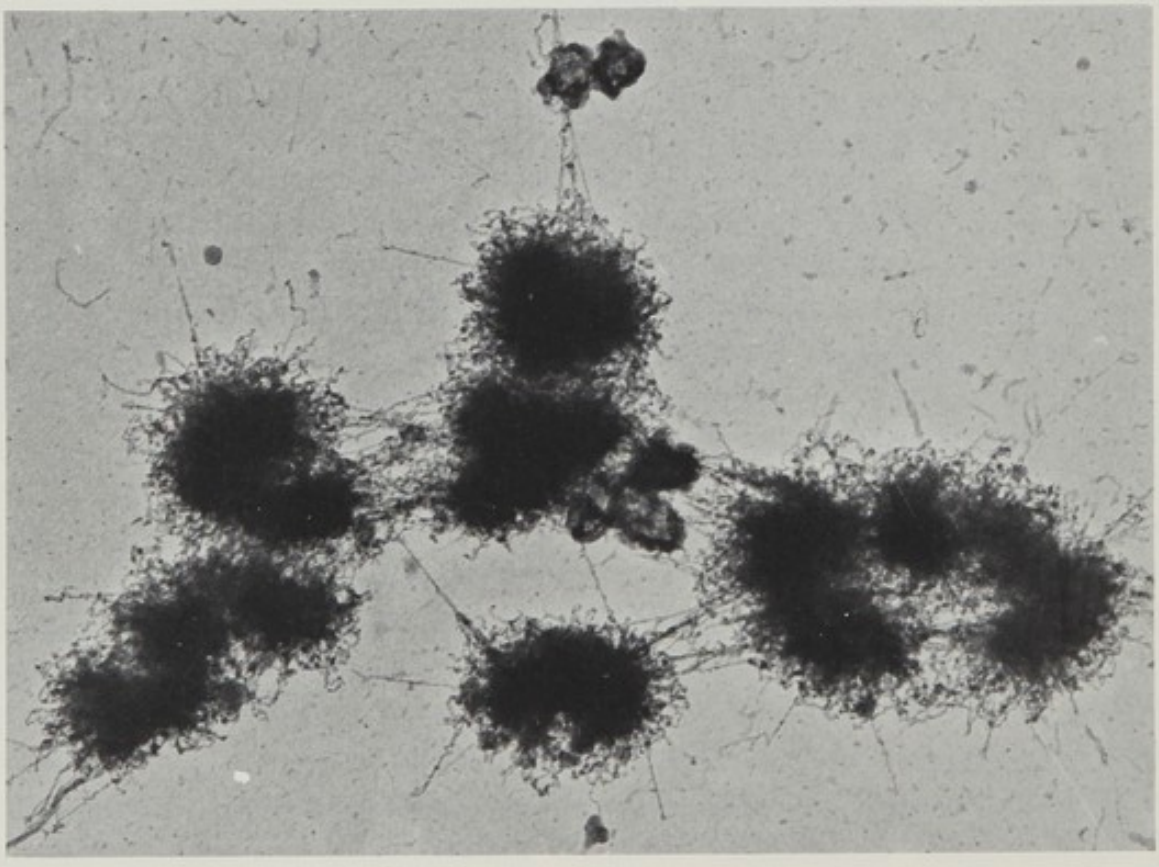
The ultrastructure of chromosomes prepared in this way can be even more precisely defined by quantitative techniques of electron microscopy (Zeitler and Bahr, 1962; Bahr and Zeitler, 1965; DuPraw and Bahr, 1969). In this method the electron scattering properties of an object are compared with the electron scattering of standard objects such as small polystyrene spheres. This electron scattering is measured by the density of silver grains exposed in a photographic plate, as determined by a scanning or integrating photometer. By applying these techniques, it has recently proved possible to measure quantitative properties such as the total dry mass of chromatids, or the length and arrangement of fibers within a chromatid; such measurements have been made both for normal human chromosomes (DuPraw and Bahr, 1969) and abnormal ones (Lampert *et al.*, 1969).

1. *Ultrastructure of Metaphase Chromosomes from Burkitt's Lymphoma, as Seen by Whole-Mount Electron Microscopy*

With the kind help of Alan S. Rabson, M. D. (National Cancer Institute, Bethesda, Md.), we obtained a Burkitt's lymphoma cell line derived from the jaw tumor of an African boy and serially propagated since 1964 in suspension culture. In this well-established AL-1 cell line, a large acrocentric marker chromosome was consistently present (Stewart *et al.*, 1965; Rabson *et al.*, 1966). During the first years of culture, the modal chromosome number in these cells was 46, but lately a more pronounced aneuploidy has appeared with chromosome numbers ranging from 44 to 52 and a modal number of 48 (Lampert *et al.*, 1969). Chromosomes from these cultured lymphoblasts were studied by whole-mount electron microscopy after isolation by surface spreading and critical point drying. Grids prepared in this way usually contained one to several separate groups of metaphase chromosomes and a few interphase nuclei.

Even at low magnification, some important chromosome features can be seen (Fig. 6). Chromosomes in mitosis look like tightly folded bundles made up of fine fibers; after careful spreading, the chromosomes





remain connected by a few of these fibers (Fig. 7). It is striking to recognize with the electron microscope a multiple association known from light microscopy between acrocentric chromosomes like the large marker and a pair of 13–15 chromosomes. Mechanical attachment by a few fibers (Fig. 7), as well as their common function as nucleolar organizers in human cells (Ohno *et al.*, 1961), seems to favor the juxtaposition of acrocentric chromosomes even after spreading. Sometimes chromosomes prepared by surface spreading–critical point drying are so loosely organized that they are hard to identify; nevertheless, in these chromosomes one can look straight through the decondensed chromatids and observe that no internal cores or subdivision into half or quarter chromatids are visible (Fig. 8). The chromatids are composed entirely of fibers varying around 250 Å in diameter. Although broken ends of fibers are often seen in the chromatids, these are probably due to the tearing process involved in spreading; the ultrastructure gives a strong impression that one, long irregularly folded fiber generates one chromatid (DuPraw, 1965, 1968).

Structures which might be mistaken for cores are sometimes produced artificially by melting together of fibers lying in the midst of each chromatid. Very likely long exposure to a chelating agent, such as sodium citrate, or other external factors may produce this effect. Correct karyotype identification of such chromosomes is often easier (Fig. 9). The large acrocentric marker chromosome, which evidently arose during the malignant transformation to Burkitt's tumor cells, does not exhibit any gross difference in its fiber substructure (Fig. 10). In this illustration, the elastic properties of the fibers can be visualized by the different positions of the two sister chromatids: one is relatively contracted, while the other one is stretched and exhibits some macrocoiling.

2. *Comparison of Chromosome Dry Mass in Cultured Human Normal and Malignant Cells*

Chromosomes from these Burkitt's lymphoma cells, as well as from short-term blood cultures from normal individuals, were further investigated by quantitative electron microscopy. The photometric procedure for measuring absolute dry mass values of whole chromosomes and their fibers have been described in detail by DuPraw and Bahr (1969), Lampert *et al.* (1969), and DuPraw (1970).

FIG. 6. Whole-mount electron microscopy of metaphase chromosomes from Burkitt's lymphoma cell line AL-1 after critical point drying. The chromosomes look like bundles of tightly folded fibers (top), sometimes showing complex interchromosomal connections (bottom). *Top:* 5000×; *bottom:* ca. 15,000×.

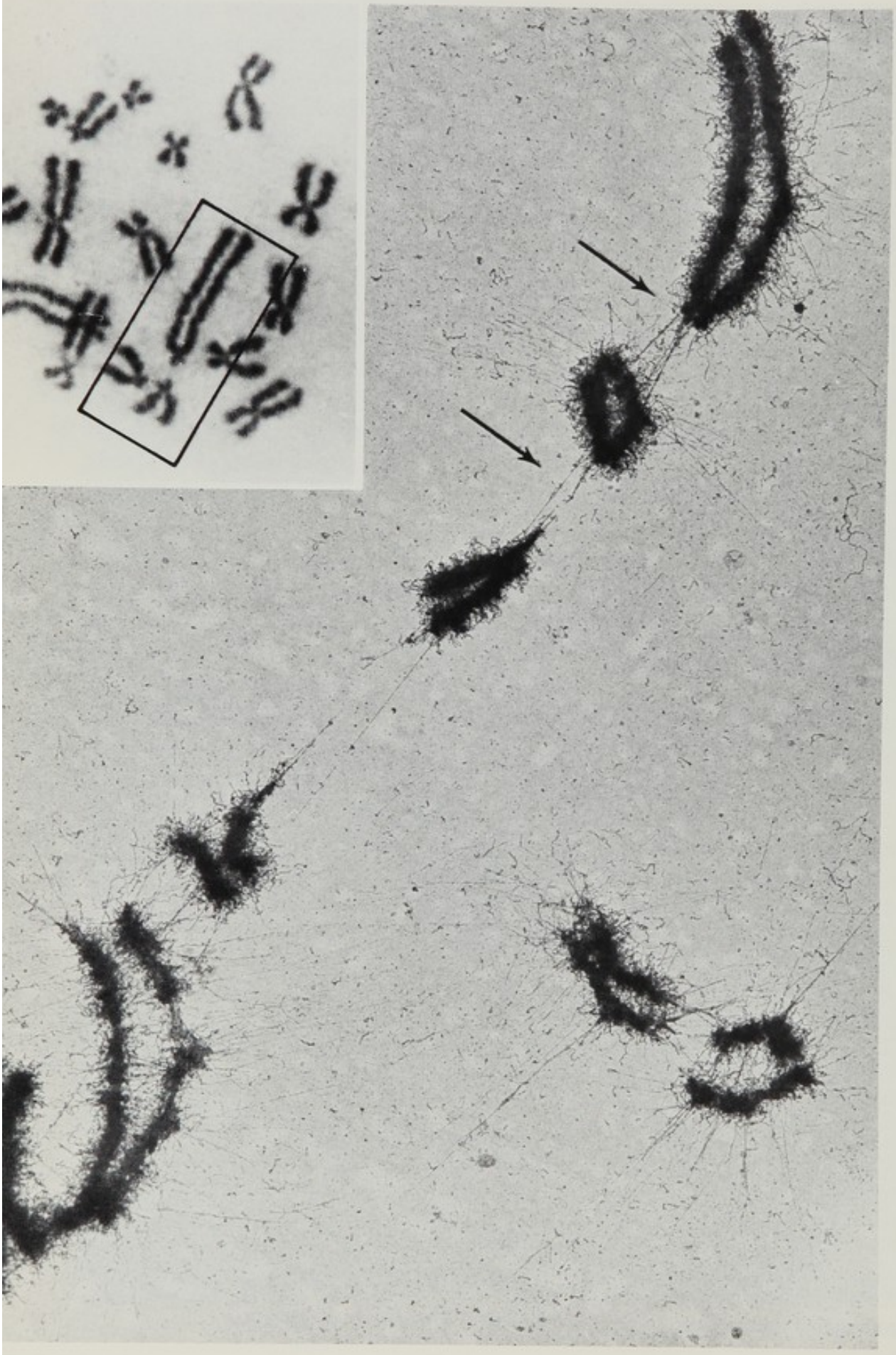


Table I summarizes our measurements of 54 chromosomes from phytohemagglutinin stimulated normal blood lymphocytes and 88 chromosomes from cultured Burkitt's tumor cells. Dry mass values are expressed in absolute units of 10^{-13} gm. Only clearly identifiable chromosomes were used, but these were measured in different metaphases and from different preparations. After pooling the values for each chromosome group, no significant difference in mean dry mass between normal and malignant cell chromosomes was found. For example, chromosomes from group 13–15 had an average weight of 12.4×10^{-13} gm, regardless whether they were derived from normal cells or from a malignant cell line. Dry mass determinations proved useful in further characterizing the long acrocentric marker and in inferring its origin in the normal karyotype; specifically, the average dry mass of the marker chromosome (23.4×10^{-13} gm) was very close to the average dry mass of a normal chromosome No. 2 (24.6×10^{-13} gm), while at the same time a normal No. 2 was missing from the karyotype.

Absolute weights of homologous chromosomes differed somewhat from metaphase to metaphase, but within each metaphase the relationship in dry mass between chromosomes from different groups was always constant. There was always a factor of about 5 between the largest (No. 1) and the smallest (No. 21–22) chromosome. This is in good agreement with other quantitative measurements of the human karyotype; for example, in percent area of the complete karyotype, the largest chromosome (4.4%) and the smallest (0.8%) differ by a factor of 5.5 (Lampert, 1967); in percent DNA content the relation between a No. 1 (8.38%) and a No. 21–22 (1.64%) is 5.12 (Rudkin, 1967). Measurements of the dry mass distribution between the two arms of a given chromatid further confirm the proportionality between dry mass and DNA content; thus the fractional dry mass in the long arms of chromosomes 1 (53%), 4–5 (70%), 13–15 (82%), 16 (60%), and 21–22 (79%) is almost identical to the fractional DNA content of the long arms in these chromosomes (53%, 70%, 83%, 60%, and 79%), as measured by Rudkin (1967; see also DuPraw, 1970).

3. *Ultrastructure of Chromosomal Fibers*

The main structural element of a chromosome is a long irregularly folded fiber. This fiber is not straight with a regular diameter like a

FIG. 7. A large acrocentric marker chromosome from Burkitt's lymphoma cell line AL-1 connected to two 13–15 chromosomes, as seen by light (1500×) and by whole-mount electron microscopy (6000×).

FIG. 8. Well spread and partially disrupted chromatids of a chromosome from cell line AL-1. The fibers represent the entire structure of the chromatids. 22,000×.

cylinder, but is bumpy like a twisted segment of spaghetti. Consequently, the diameters of critical point dried fibers range on the average between 200 and 400 Å. After critical point drying, electron microscopy does not reveal much detail regarding the internal architecture of this fiber, even at higher magnifications (Fig. 11). Although a fine stripe of higher electron density can sometimes be observed running along the center of the fiber, one has to be careful in interpreting this because of the ease with which fibers become contaminated in the electron beam.

Thin sections of chromosomes from the same cells, which were fixed and embedded by the usual glutaraldehyde–osmium–Epon techniques, at first sight seem to exhibit only electron dense material of granular

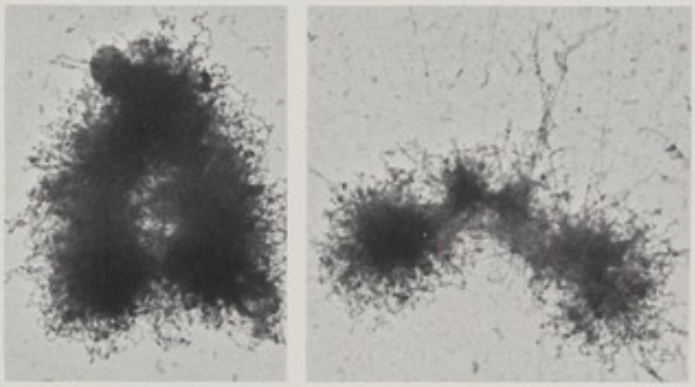


FIG. 9. A chromosome 13–15 and a chromosome 21–22 from an AL-1 cell. "Melted" fibers in the center of the chromatids can be mistaken for a core. 13,900×.

and fibrillar elements (Fig. 12). However, with careful study, one can see fibers about 200 Å in diameter (Fig. 12, black arrow), which seem to be composed of a smaller fiber less than 100 Å in diameter arranged in a supercoiled configuration (Fig. 12, white arrow). The detection of supercoiling in thin-sectioned chromosomes further supports the folded fiber model of DuPraw (1965, 1970), who postulated that a first-order DNA supercoil is packed into a 50–110 Å (type A) chromatin fiber, which then undergoes second-order supercoiling to generate a coiled-coil 200–300 Å (type B) fiber. X-ray diffraction patterns from chromatin fibers also suggest a superhelical model for nucleohistone (Pardon *et al.*, 1967). At the present time, however, qualitative electron microscopic techniques alone probably can not resolve the internal fiber structure convincingly. Fortunately, dry mass determinations favor a very tight arrangement of DNA within the chromosome fiber and thus quantify the subjective morphological conclusions.

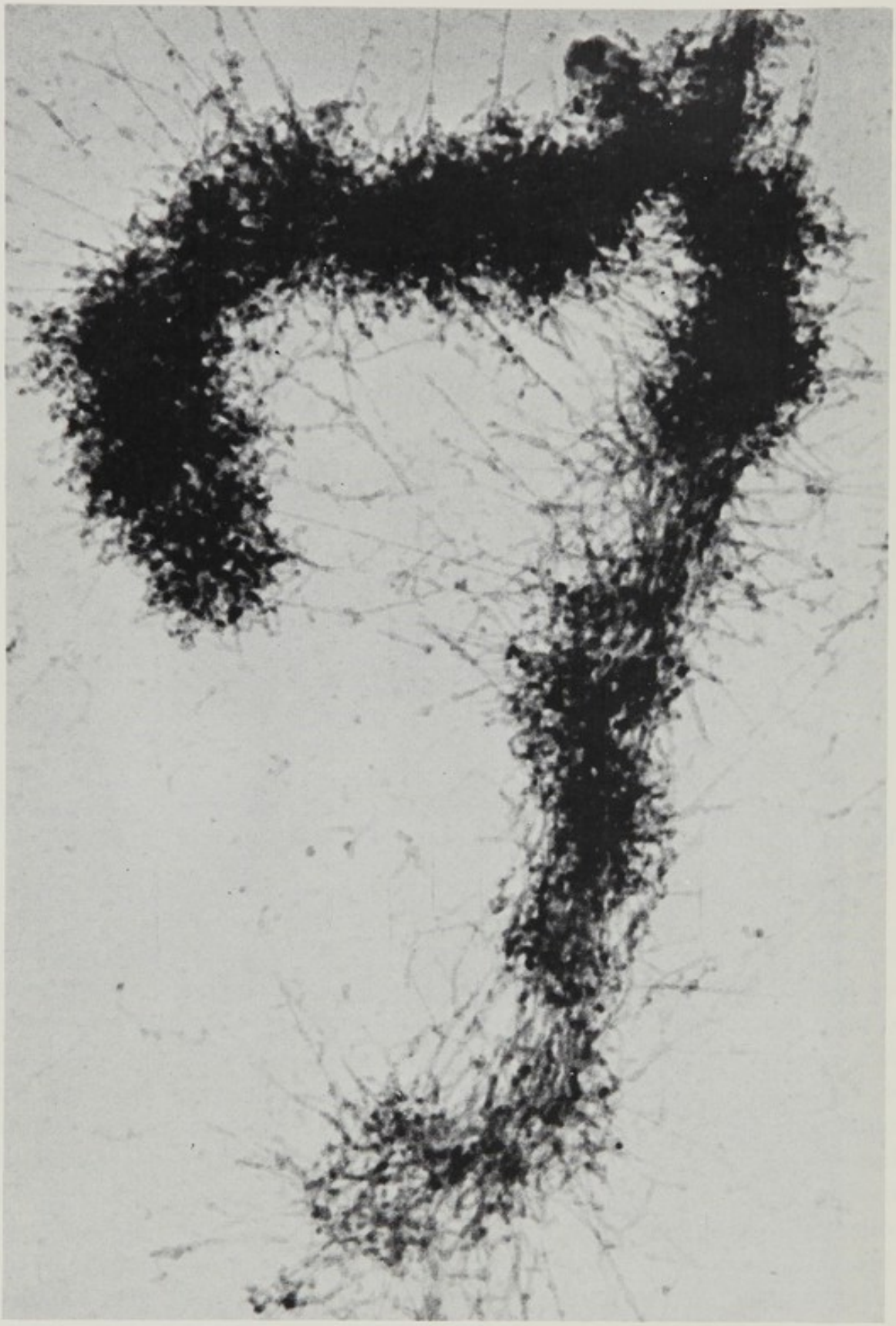


FIG. 10. A large acrocentric marker chromosome from Burkitt's lymphoma cell line AL-1 after surface spreading and critical point drying. Notice one stretched and one contracted chromatid; chromatid macrocoiling is visible after stretching. 30,100×.

TABLE I

DRY MASS (M) OF HUMAN CHROMOSOMES, AS DETERMINED BY QUANTITATIVE ELECTRON MICROSCOPY[a]

Chromosome	% M in long arm	M in relation to 21–22	Preparation from normal blood lymphocytes			Preparation from Burkitt's tumor cells (cell culture AL-1)		
			M (10^{-13} gm)	σ	n	M (10^{-13} gm)	σ	n
1	52	5.7	32.5	±10.8	5	—	—	—
2	55	4.3	24.6	±3.4	2	23.4	±2.7 ("Marker")	5
3	53	3.7	22.4	±3.6	2	20.2	—	1
4–5	70	3.1	14.9	±2.1	7	19.5	±2.2	2
6–X–12	64	2.9	16.1	±4.9	14	16.9	±3.0	23
13–15	82	2.1	12.4	±3.7	7	12.4	±2.3	16
16	60	1.8	11.9	±2.5	2	10.6	±1.7	5
17–18	64	1.6	9.1	±1.4	3	9.6	±1.9	10
19–20	56	1.4	7.1	±0.9	5	8.9	±2.0	9
Y–21–22	79	1.0	5.7	±1.4	7	5.8	±1.0	17

[a] Standard deviation, σ; number, n.

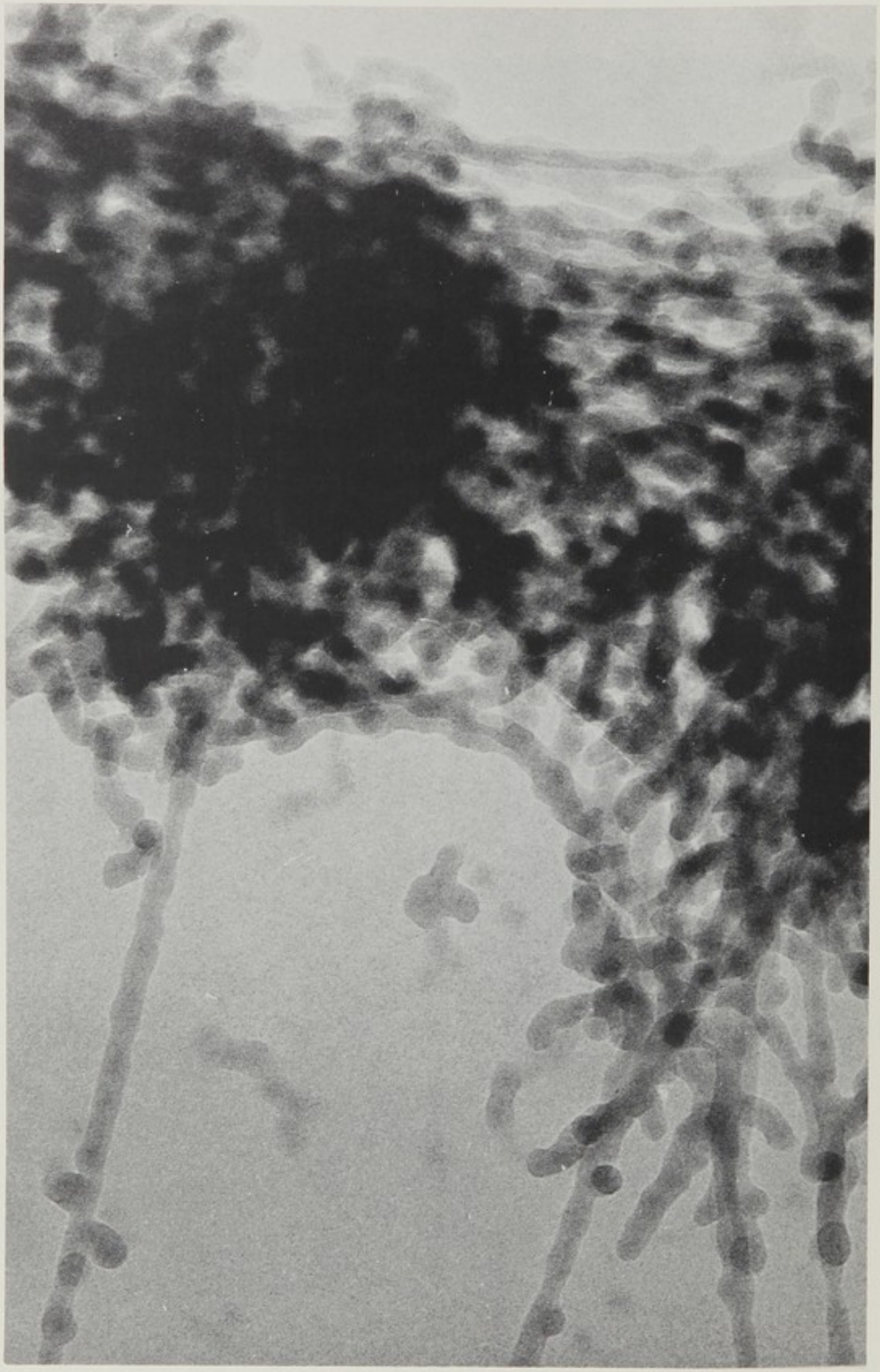


FIG. 11. Fibers of the centromere region in an acrocentric chromosome from an AL-1 cell. No definite detail can be seen within the bumpy fibers. 110,000×.

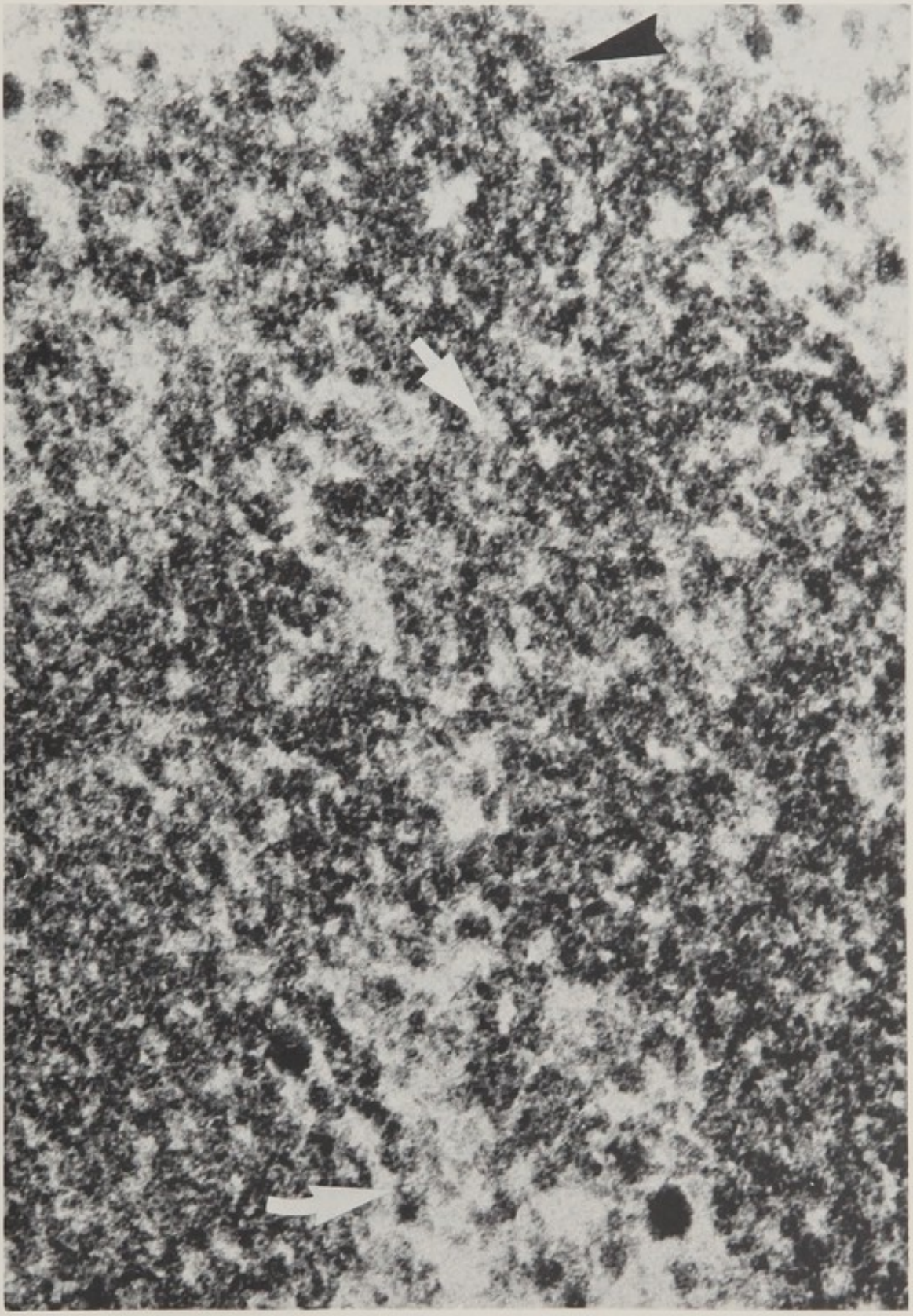


FIG. 12. Longitudinal thin section through the centromere region of an acrocentric chromosome from an AL-1 cell. The fibers (black arrow) seem to be composed of much thinner subfibers which are tightly supercoiled (white arrow). Glutaraldehyde–osmium–Epon. 160,000×.

4. *Dry Mass of Human Chromosome Fibers from Cultured Normal and Malignant Cells*

The dry mass of fibers in metaphase chromosomes from normal blood lymphocytes and from Burkitt's lymphoma cells was measured in 500 to 1000 Å long segments; these were chosen because they protruded from the chromatids and had a fairly uniform diameter. Measured data were then normalized for a fiber length of 1 micron. In Table II, the dry mass, diameter, and calculated density of 59 fiber segments are summarized.

The absolute dry mass per micron of fiber has an order of magnitude of 10^{-16} gm and tends to be proportional to fiber diameter. However, unusually large diameters of critical point dried fibers should be regarded with caution because longer exposure to the electron beam at

TABLE II

DRY MASS (M/μ), DIAMETER (d), AND DENSITY (ρ) OF HUMAN CHROMOSOME FIBERS

Cell type	M (10^{-16} gm/μ) $\pm$ σ	d (Å) $\pm$ σ	ρ (gm/cm^3)	n
Normal blood lymphocytes	6.9 $\pm$ 2.6	254 $\pm$ 53	1.36	20
Burkitt's tumor (cell culture AL-1)	11.2 $\pm$ 1.6	333 $\pm$ 66	1.29	39

high magnification can cause artificial enlargement by contamination (Gall, 1966). For this reason, we hesitate to emphasize the significant difference ($P < 0.001$) found in average dry mass between fibers from normal (6.9×10^{-16} gm/μ) and tumor chromosomes (11.2×10^{-16} gm/μ). Nevertheless, if the individual data for fiber dry mass (Y) are plotted against the squares of fiber diameters (X), then a line fitted to the points by the method of least squares has the formula $Y = 3.0 + 0.69\ X$ for Burkitt's tumor chromosomes (Lampert *et al.*, 1969) and $Y = 2.0 + 0.78\ X$ for chromosomes from blood lymphocytes (DuPraw and Bahr, 1969). When the most frequent fiber diameter of 250 Å is substituted into these formulas, we get expected dry masses of 7.37 and 6.87×10^{-16} gm per micron of fiber, respectively. The value for normal chromosomes is very close to our measured average fiber weight of 6.9×10^{-16} gm/μ. If these fibers are regarded as cylinders, then density (specific gravity) can be calculated from dry mass per micron divided by πr^2; the calculated densities of 1.36 and 1.29 gm/cm^3 for

our measured fiber segments lie around 1.31 gm/cm^3, a value which has been determined by sucrose gradient centrifugation for bulk isolated HeLa cell chromosomes (Huberman and Attardi, 1967). Furthermore, as pointed out by DuPraw and Bahr (1969), a density of about 1.3 gm/cm^3 is to be expected for structures which, like isolated chromosomes, are composed of 16–20% DNA (density 1.7) and 60–75% protein (density 1.25) (Huberman and Attardi, 1966; Salzman *et al.*, 1966; Maio and Schildkraut, 1967).

Knowing the dry mass of an extended DNA double helix (3.26×10^{-18} gm/μ), and assuming a DNA percentage of 20% in a chromosome fiber which has a mass of 7×10^{-16} gm/μ, we can calculate that about 43 μ of DNA have to be packed into 1 μ of this chromosome fiber ($0.2 \times 7 \times 10^{-16}/3.26 \times 10^{-18}$). This high packing ratio of DNA in chromosome fibers is only possible by means of supercoiling. This conclusion has been checked by estimating the amount of DNA, i.e., the length of DNA-double helix per individual chromosome of the human karyotype, and then calculating individual DNA-packing ratios after determination of the total length of type B fiber per chromosome (DuPraw and Bahr, 1969). These DNA-packing ratios vary in individual chromosomes, depending on the degree of contraction during mitosis. However, DNA-packing ratios greater than 100:1 have been measured in fibers from individual normal chromosomes (DuPraw and Bahr, 1969). DNA-packing ratios as high as this have not been observed in chromosomes from Burkitt's lymphoma cells, especially in the marker chromosome (Lampert *et al.*, 1969). One might speculate, therefore, that chromosome fibers from malignant cells are altered and that the changed relationship of DNA to protein disturbs their elastic behavior, leading to fiber breakage or altered folding patterns. Many disturbances of the karyotypes of tumor cells could then be explained.

D. Causes of Chromosome Alterations

As discussed in an article by Kihlman in this volume, chromosome alterations can be induced *in vivo* and *in vitro* by ionizing radiation (Bender and Gooch, 1962; Grouchy *et al.*, 1963b; Wald *et al.*, 1964; Cole and Nowell, 1965), chemicals (Levan and Tjio, 1948; Taylor *et al.*, 1962; Kihlman *et al.*, 1963; Bell and Wolff, 1964; Brewen, 1965), and viruses (Boiron *et al.*, 1966; MacKinnon *et al.*, 1966; Aula *et al.*, 1968). Each of these external factors is also able to transform normal cells into malignant cells.

At present carcinogenesis by viruses has become the most discussed

model (Dulbecco, 1967; Habel, 1968; Temin, 1969). The inducing or transforming factor in viral carcinogenesis is thought to be the viral DNA, which persists in transformed cells (Westphal and Dulbecco, 1968) and is integrated into the cell's own chromosomes (Sambrook *et al.*, 1968; Weiss *et al.*, 1968). Persistence of chromosomal instability in neoplastic cells might be explained by a similar retention of a viral genome or part of it (Stoltz *et al.*, 1967). In human cancers, the presence of herpes-type viruses has been detected in cells of Burkitt's lymphoma and acute leukemia (Epstein *et al.*, 1964); this Epstein-Barr virus is also able to transform normal human leukocytes *in vitro* (Gerber *et al.*, 1969), and it produces chromosome changes such as breaks, secondary constrictions, polyploidy, and finally pulverization. Quite possibly virus particles may cause both chromosomal abnormalities and cancer in man.

For these reasons, we will understand the nature of cancer only after we have explored the molecular events controlling cell differentiation and gene regulation. The process of gene regulation, in turn, must certainly depend on the structural arrangement of DNA within the chromosome fibers. The discovery of high DNA-packing ratios in these fibers and the variation in packing ratio between normal and malignant cell chromosomes provides useful insights in this direction.

REFERENCES

Anderson, T. F. (1951). *Trans. N.Y. Acad. Sci.* [2] **13**: 130.
Atkin, N. B. (1967). *Eur. J .Cancer* **3**: 289.
Atkin, N. B., and Baker, M. C. (1966). *J. Nat. Cancer Inst.* **36**: 539.
Aula, P., Nichols, W. W., and Levan, A. (1968). *Ann. N.Y. Acad. Sci.* **155**: 737.
Bahr, G. F., and Zeitler, E. (1965). *Lab Invest.* **14**: 955.
Bell, S., and Wolff, S. (1964). *Proc. Nat. Acad. Sci. U.S.* **51**: 195.
Bender, M. A., and Gooch, P. C. (1962). *Proc. Nat. Acad. Sci. U.S.* **48**: 522.
Boiron, M., Tanzer, J., Thomas, M., and Hampe, A. (1966). *Nature* **209**: 737.
Boveri, T. H. (1914). "Zur Frage der Entstehung maligner Tumoren." Fischer, Jena.
Brandão, H. J. S., and Atkin, N. B. (1968). *Brit. J. Cancer* **22**: 184.
Brewen, J. G. (1965). *Cytogenetics* **4**: 28.
Castro-Sierra, E., Gorman, L. Z., and Merker, H. (1967). *Humangenetik* **4**: 62.
Clein, G. P., and Flemans, R. J. (1966). *Brit. J. Haematol.* **12**: 754.
Cole, L. J., and Nowell, P. C. (1965). *Science* **150**: 1782.
Conen, P. E., and Erkman, B. (1966). *Amer. J. Dis. Child.* **112**: 429.
Dulbecco, R. (1967). *Sci. Amer.* **216**: 28.
DuPraw, E. J. (1965). *Proc. Nat. Acad. Sci. U.S.* **53**: 161.
DuPraw, E. J. (1966). *Nature* **209**: 577.
DuPraw, E. J. (1968). "Cell and Molecular Biology." Academic Press, New York.
DuPraw, E. J. (1970). "DNA and Chromosomes." Holt, New York.
DuPraw, E. J., and Bahr, G. F. (1969). *Acta Cytol.* **13**: 188.
Epstein, M. A., Achong, B. G., and Barr, Y. M. (1964). *Lancet* **I**: 702.

Evans, H. J. (1967). *Nature* **214**: 361.
Fitzgerald, P. H. (1966). *J. Med. Genet.* **3**: 258.
Gall, J. G. (1966). *Chromosoma* **20**: 221.
Gerber, P., Whang-Peng, J., and Monroe, J. H. (1969). *Proc. Nat. Acad. Sci. U.S.* **63**: 740.
German, J., Archibald, R., and Bloom, D. C. (1965). *Science* **148**: 506.
Goh, K. (1968). *Arch. Intern. Med.* **122**: 241.
Grouchy, J. de, Vallee, G., and Lamy, M. (1963a). *C. R. Acad. Sci.* **256**: 2046.
Grouchy, J. de, Vallee, G., and Lamy, M. (1963b). *C. R. Acad. Sci.* **257**: 278.
Grouchy, J. de, Nava, C. de, Cantu, J. M., Bilski-Pasquier, G., and Bousser, J. (1966). *Amer. J. Hum. Genet.* **18**: 485.
Habel, K. (1968). *Cancer Res.* **28**: 1825.
Hecht, F., Koler, R. D., Rigas, D. A., Dahnke, G. S., Case, M. P., Tisdale, V., and Miller, R. W. (1966). *Lancet* **II**: 1193.
Huberman, J. A., and Attardi, G. (1966). *J. Cell Biol.* **31**: 95.
Huberman, J. A., and Attardi, G. (1967). *J. Mol. Biol.* **29**: 487.
Ishihara, T., Moore, G. E., and Sandberg, A. A. (1961). *J. Nat. Cancer Inst.* **27**: 893.
Ishihara, T., Kikuchi, Y., and Sandberg, A. A. (1963). *J. Nat. Cancer Inst.* **30**: 1303.
Jacobs, E. M., Luce, J. K., and Cailleau, R. (1966). *Cancer* **19**: 869.
Jacobs, P. A., Tough, I. M., and Wright, D. H. (1963). *Lancet* **II**: 1144.
Kemp, N. H., Stafford, J. L., and Tanner, R. (1964). *Brit. Med. J.* **1**: 1010.
Kihlman, B. A., Nichols, W. W., and Levan, A. (1963). *Hereditas* **50**: 139.
Kleinschmidt, A. K., Lang, D., and Zahn, R. K. (1959). *Z. Naturforsch.* **14b**: 770.
Koller, P. C. (1947). *Brit. J. Cancer* **1**: 38.
Kosenow, W., and Pfeiffer, R. A. (1969). *Deut. Med. Wochenschr.* **94**: 1170.
Krivit, W., and Good, R. A. (1957). *Amer. J. Dis. Child.* **94**: 289.
Lampert, F. (1967). *Klin. Wochenschr.* **45**: 512.
Lampert, F. (1968). *Virchow's Arch. B* **1**: 31.
Lampert, F. (1969a). *Deut. Med. Wochenschr.* **94**: 217.
Lampert, F. (1969b). *Haematol. Bluttransf.* **8**: 108.
Lampert, F., Karsch, P., and Goldenberg, D. M. (1968). *Arch. Geschwulstforsch.* **32**: 309.
Lampert, F., Bahr, G. F., and DuPraw, E. J. (1969). *Cancer* **24**: 367.
Levan, A., and Tjio, J. H. (1948). *Hereditas* **34**: 453.
MacKinnon, E., Kalnins, V. I., Stich, H. F., and Yohn, D. S. (1966). *Cancer Res.* **26**: 612.
Maio, J. J., and Schildkraut, C. L. (1967). *J. Mol. Biol.* **24**: 29.
Makino, S. (1957). *Int. Rev. Cytol.* **6**: 26.
Makino, S., Ishihara, T., and Tonomura, A. (1959). *Z. Krebsforsch.* **63**: 184.
Makino, S., Sasaki, M. S., and Tonomura, A. (1964). *J. Nat. Cancer Inst.* **32**: 741.
Miles, C. P. (1967). *Cancer* **20**: 1253, 1274.
Nowell, P. C., and Hungerford, D. A. (1960). *Science* **132**: 1497.
Ohno, S., Trujillo, J. M., Kaplan, W. D., and Kinosita, R. (1961). *Lancet* **II**: 123.
Pardon, J. F., Wilkins, M. H. F., and Richards, B. M. (1967). *Nature* **215**: 508.
Peterson, R. D. A., Cooper, M. D., and Good, R. A. (1966). *Amer. J. Med.* **41**: 342.
Rabson, A. S., O'Conor, G. T., Baron, S., Whang, J. P., and Legallais, F. Y. (1966). *Int. J. Cancer* **1**: 89.

Rigby, C. C. (1968). *Brit. J. Cancer* **22**: 480.
Ris, H., and Chandler, B. L. (1963). *Cold Spring Harb. Symp. Quant. Biol.* **28**: 1.
Rudkin, G. T. (1967). *In* "The Chromosome" (G. Yerganian, ed.), p. 12. Williams & Wilkins, Baltimore, Maryland.
Rudkin, G. T., Hungerford, D. A., and Nowell, P. C. (1964). *Science* **144**: 1229.
Salzman, N. P., Moore, D. E., and Mendelsohn, J. (1966). *Proc. Nat. Acad. Sci.* U.S. **56**: 144.
Sambrook, J., Westphal, H., Srinivasan, P. R., and Dulbecco, R. (1968). *Proc. Nat. Acad. Sci. U.S.* **60**: 1288.
Sandberg, A. A. (1966). *Cancer Res.* **26**: 2064.
Sandberg, A. A., Bross, I. D. J., Takagi, N., and Schmidt, M. L. (1968a). *Cancer* **21**: 77.
Sandberg, A. A., Takagi, N., Sofuni, T., and Crosswhite, L. H. (1968b). *Cancer* **22**: 1268.
Schroeder, T. M., Anschütz, F., and Knopp, A. (1964). *Humangenetik* **1**: 194.
Stark, C. R., and Mantel, N. (1966). *J. Nat. Cancer Inst.* **37**: 687.
Stewart, A., Webb, J., and Hewitt, D. (1958). *Brit. Med. J.* **1**: 1495.
Stewart, S. E., Lovelace, E., Whang, J. J., and Ngu, V. A. (1965). *J. Nat. Cancer Inst.* **34**: 319.
Stoltz, D. B., Stich, H. F., and Yohn, D. S. (1967). *Cancer Res.* **27**: 587.
Taylor, J. H., Haut, W. F., and Tung, J. (1962). *Proc. Nat. Acad. Sci. U.S.* **48**: 190.
Temin, H. M. (1969). *Radiology* **92**: 931.
Tough, I. M., Jacobs, P. A., Court-Brown, W. M., Baikie, A. G., and Williamson, E. R. D. (1963). *Lancet* **I**: 844.
Wald, N., Upton, A. C., Jenkins, V. K., and Borges, W. H. (1964). *Science* **143**: 810.
Weiss, M. C., Ephrussi, B., and Scaletta, L. J. (1968). *Proc. Nat. Acad. Sci. U.S.* **59**: 1132.
Westphal, H., and Dulbecco, R. (1968). *Proc. Nat. Acad. Sci. U.S.* **59**: 1158.
Whang, J. P., Frei, E., III, Tjio, J. H., Carbone, P. P., and Brecher, G. (1963). *Blood* **22**: 664.
Whang, J. P., Canellos, G. P., Carbone, P. P., and Tjio, J. H. (1968). *Blood* **32**: 755.
Yamada, K., Takagi, N., and Sandberg, A. A. (1966). *Cancer*, **19**: 1879.
Zeitler, E., and Bahr, G. F. (1962). *J. Appl. Phys.* **33**: 847.

ARCHITECTURE AND FUNCTION OF THE MITOTIC SPINDLE

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I. Introduction

Microtubules as components of the mitotic spindle were described by de Harven and Bernhard (1956). However, understanding of spindle fine structure increased greatly after Ledbetter and Porter (1963)

stressed their importance, and clarification of several important questions followed. A review by Buvat (1963) covered early electron microscope data on plant material. The role of centrioles has been discussed in very stimulating reviews by Stubblefield and Brinkley (1968) and by Pickett-Heaps (1969d). Luykx (1970) has extensively reviewed the structure and function of the spindle and kinetochores from light and electron microscopy and a genetic point of view. Gibbons (1968) has discussed the structure of flagella and cilia, and Stephens (1970) has reviewed the latest data on the chemical composition of microtubules (MTs).

It is evident from these reviews that there are still several unclear points in relation to the microtubules' composition and assembly during mitosis, e.g., as raised by the work of Forer and Goldman (1969) and by Bibring and Baxendall (1969). This is also stressed in a critical discussion of literature on the spindle and microtubules by Newcomb (1969). Therefore, it will not be the purpose of this article to present a comprehensive review of the literature, but instead to correlate some events during mitosis and cytokinesis as known from classic studies using the light microscope, with present data on fine structure.

It is apparent from the literature that important facts are often forgotten or overlooked in contemporary attempts to interpret the mechanism of mitosis. This leads to oversimplifications, as evident in some new hypotheses, which only increase the confusion in the literature. One of the main difficulties in interpretation is that data are obtained using a variety of species, whereas extrapolations from one species to another may lead to serious error.

Several aspects of spindle structure and activity in different stages of mitosis will be described. To avoid possible misunderstandings, such expressions as *pulling*, *pushing*, *elimination*,° and *transport* will be used here in purely descriptive form. When the chromosome bends poleward at the kinetochore region and moves toward the pole, it is described as being "pulled" poleward. When the kinetochore is nearly or completely stationary (as in metaphase of *Haemanthus*), but the long chromosome arms move to the pole or outside the spindle (e.g., in the newt), it will be said that the arms are eliminated toward the poles or out of the spindle. Elimination is especially clear with respect to objects unattached to the spindle (small granules, acentric fragments, etc.), but it is often difficult to decide whether an object is "eliminated" or "pushed out." The difference implies different mechanisms and may

° The problem of *elimination* has been simplified here. There are different types of elimination operated by different mechanisms. This problem, however, will not be discussed here (see Bajer and Molè-Bajer, 1971).

be essential: for example, elimination or transport undoubtedly occurs very close to MTs (Bajer, 1968b), while pushing away may be the ride on the tips of growing (elongating) MTs or the ride of trapped material between MTs due to growth of the MTs themselves (see Section XIII). Sometimes elimination and pulling occur simultaneously in the spindle (e.g., in prometaphase), resulting in movements toward opposite directions by objects very close to one another. These properties of the spindle (and most probably asters) are considered to be its most important feature and, in fact, may reflect the basic activity of MTs in living cells. The capability for simultaneous transport toward and away from the poles is a basic feature of mitotic spindles which should always be kept in mind to avoid oversimplification. This feature produces rather complicated transport directions within the spindle which are schematically represented in Fig. 4. The problem of transport has been thoroughly reviewed in previous articles (Bajer and Molè-Bajer, 1963; Bajer, 1968a).

Plant material, especially the endosperm of *Haemanthus katherinae*, will be discussed extensively in this article. So far, no other system has made it possible to compare the behavior of chromosomes *in vitro* with fine structure to such an extent. A large amount of informaion has been obtained with respect to the rearrangement of MTs during mitosis and this aspect will be considered most extensively.

The following abbreviations will be used throughout this article.

BNE	Breakdown of nuclear envelope	GB	Golgi body (dictyosome)
		LM	Light microscope
CS	Contraction stage	MF(s)	Microfilament(s)
CZ	Clear zone	MT(s)	Microtubule(s)
EM	Electron microscope	NE	Nuclear envelope
ER	Endoplasmic reticulum	UV	Ultraviolet

II. Techniques and Their Limitations

In order to correlate changes in living cells with their fine structure, a comparison of living cells with specimens that have been fixed, dehydrated, and embedded is especially important. Bloom (1960) was successful in locating a UV irradiated chromosome in the EM, but the technique was comparatively complicated. A much simpler method was proposed by Robbins and Gonatas (1964); this technique, using a perfusion chamber, is suitable for cells which adhere strongly to cover slips (coated with carbon), making it possible to observe the cell up to the moment of fixation and even during dehydration. The latter experi-

ments have been reported for plant but not animal material so far. A still simpler technique was used by Brinkley *et al.* (1967b), in which it was not possible to observe living cells, but they could select individual cells under the LM (after embedding in plastic) for subsequent sectioning for the EM; the plane of sectioning and the stage of the cell in mitosis were always precisely known. Individual cells which do not stick to the cover slip can be observed in a perfusion chamber if such cells are covered with a thin film of ager and gelatin, permitting penetration of fixatives (Molè-Bajer and Bajer, 1968).

Time-lapse observations of *Haemanthus* endosperm cells during actual fixation indicate that individual cells under the same experimental conditions respond to fixatives in different ways. An important question is: how long does it take to kill the cell? In the author's experiments the time is estimated as 10 to 30 seconds (perfusion chamber, single layer of cells). Inasmuch as this time is long enough for several processes to take place, e.g., chromosomes may move as much as 0.5 μ, the unavoidable conclusion is that cells have enough time to mobilize several "defense mechanisms" during fixation (e.g., a membrane system may be formed or destroyed). This may be the reason why so little is known about the "ends" of MTs, etc., because these may be more easily modified or destroyed during fixation or dehydration.

Observations of dividing cells during fixation and subsequent dehydration show that during dehydration different components of the cell shrink to a different extent, and this shrinkage is often directional, e.g., chromosome arms shrink toward the kinetochore. The latter remains stationary, evidently because the chromosome is attached firmly to the spindle at the kinetochore. A comparison of the degrees of shrinkage and lengths of MTs indicates that the occasional waviness of MTs is the result of chromosome shrinkage during dehydration (Jensen and Bajer, 1969); most likely MTs are straight during mitosis. Suggestions that waviness may be related to MT activity during mitosis (Ledbetter and Porter, 1963; Behnke and Forer, 1966) and phragmoplast formation (Cronshaw and Esau, 1968) should be reexamined. On the other hand, it is an open question whether all types of waviness can be explained by shrinkage; Cronshaw and Esau (1968) found single wavy MTs between straight ones. A detailed study might show that such MTs are in a different state of development, broken on one end, or shorter, etc.

So far MTs are the only elements of the spindle which, without doubt, are involved in chromosome movements. However, a series of experiments by Forer (1965, 1966) on the effect of UV microbeam irradiation suggests that some additional factors beyond the spindle fibers exist and that these additional factors can contribute to chromosome move-

ment. He found that chromosome movement could be stopped either with or without an associated decrease of birefringence [now found to be associated in *Haemanthus* with a decrease in the number of MTs in the irradiated region (Sato, Bajer, and Molè-Bajer, unpublished observations)]. Forer's interpretation of the latter experiments is that spindle fibers do not transmit or produce force for chromosome movement. Alternatively, it might be speculated that spindle fibers (MTs) have at least two functions: the pulling of chromosomes and the elimination of bodies unattached to the spindle. The latter hypothesis concerning spindle fibers was originally suggested by Östergren, Molè-Bajer and Bajer (1960) and modified by Subirana (1968).

It is also possible that unknown structural elements exist in the spindle which are not preserved during EM procedures. Microfibrils (MFs) have been observed in spindles of *Haemanthus* endosperm (Bajer and Molè-Bajer, 1969). However, no detailed studies have been performed on *Haemanthus* or any other species, and it is not clear how often MFs occur in mitotic spindles. Although MFs play an important role during cleavage of animal cells, they have not as yet been found in animal spindles. A system of membranes found in the spindle (ER or remnants of NE) is clearly involved in chromosome distribution only in exceptional cases, e.g., in some dinoflagellates (Kubai and Ris, 1969). More critical work on fixation and preservation of elements of the mitotic spindle is needed.

The ER often seems to change gradually into MTs, i.e., ER or a sheet of poorly defined material closes up and forms a MT (Burgess and Northcote, 1968; Bajer and Molè-Bajer, 1969). This may produce pictures of complete or incomplete MTs in cross section. Incomplete MTs are also clearly visible during reassembly of MTs after cold treatment of blood platelets (Behnke, 1967). The region around MTs also deserves special attention. Ledbetter and Porter (1963), in their original article drawing attention to MTs, noted that there are electron light spaces around MTs. It would not be surprising if changes in this region play an essential role in the transport properties of the spindle and in chromosome movements (see Section XIII). Furthermore, this region is probably severely changed during EM preparation procedures. These are certainly crucial questions, and until they are clarified the role and function of MTs in chromosome movement will not be well understood.

III. Visibility and Resolution of Microtubules with the Light Microscope

Observation of selected regions containing MTs, both with LM and EM, permits one to draw conclusions about how MTs are seen by light

microscopy. From the theory of LM image formation, both visibility (detectability) and resolution depend on the size of the object and on the contrast between the object and the background. Objects smaller than the LM limit of resolution can be seen (detected) but cannot be resolved, i.e., their size cannot be measured. Theoretically, a tubule 50 Å in diameter can be detected with LM (Beyer, 1966); assuming 0.2 μ (2000 Å) as the practical resolving power, then objects 40 times below the limit of LM resolution can be detected. In phase contrast, a structure as thin as 500 Å (approximately the thickness of the NE) is easily seen, and this is only two times thicker than the diameter of a MT, approximately four times below LM resolution. In dark field or semidark field, objects 20 times below the limits of LM resolution can easily be seen.

Although efforts to detect single MTs with LM have so far been unsuccessful, these calculations show that the diameter of a single MT is well above the detecting limit of the LM. Three or more (5–7) MTs, if located close together, are easily seen as a distinct fiber in the LM, provided they have no dense accumulation of MTs as a background (Bajer and Jensen, 1969). Stages of cell division in which this can be observed are the early stage of phragmoplast formation in plants, and probably in animals an early stage of mid-body formation. Although the fine structure of well advanced mid-bodies has been studied extensively (see Section X,B), the earlier stages have not yet been described. When a larger number of MTs form a fibril (e.g., in *Haemanthus* a kinetochore fiber may be composed of 150 MTs), a very complicated diffraction pattern is observed and any conclusion from such a pattern has to be drawn cautiously (Allen *et al.*, 1969). However, understanding of such pattern may elucidate transport properties within the spindle.

IV. Spindle Types

The shape of the spindle is influenced by the presence or absence of polar centrioles or blepharoplasts, as well as by mechanical conditions in the cell. Consequently, astral and anastral types of spindle are commonly distinguished.

A. Anastral Spindle (Spindle without Centrioles, Blepharoplasts, or Asters)

1. *Shape of the Spindle*

A spindle of this type, as seen with the LM, is typical for higher plants. However, anastral spindles are also found in a variety of animals,

e.g., in several animal ova and in many protista (Wilson, 1928). Within the same species, an anastral spindle can change during development into an astral one or vice versa. Although in most cases the spindle has two symmetrical half-spindles, in some types of division the spindle has nonsymmetrical poles, i.e., one diffuse and one pointed. Precise definition of the spindle poles in anastral spindles is not easy, and probably the term "polar region" rather than spindle pole would be more appropriate. In asymmetric anastral spindles, the chromosomes of one group may converge toward the spindle pole while others diverge toward the opposite pole; such divisions resemble monopolar mitosis.

Asymmetric spindles are found in dividing pollen and in stomatal complexes (Pickett-Heaps, 1969a,b,c). Heslop-Harrison (1968) described very interesting pollen divisions in orchids, where the microspores do not separate but form aggregates (massulae) in which division is highly synchronous. Each spindle is pointed at the vegetative pole and rather diffuse at the generative pole. As judged from the published description, the diffuse pole becomes pointed during anaphase and a dense amorphous body about 0.6 μ in diameter is found at this pole close to the cell wall. Typical anastral spindles often have wide dome-shaped poles during metaphase, but the shape becomes more pointed during prometaphase, shortens in metaphase, and again becomes more pointed during spindle elongation in anaphase. However, during meiosis in plants the spindle is often pointed throughout the whole cycle. The decrease in length during metaphase is slight as compared with prometaphase (usually less than one fifth) and any increase in length during anaphase is also slight (usually less than one third of the metaphase spindle). However, the change in spindle shape during mitosis reflects a change in the arrangement of the MTs. MTs are comparatively stiff, and in cells without rigid walls they may occasionally push out part of the cell membrane (Bajer, 1968a).

An important conclusion is that spindles may vary in the shape and structure of the polar region, even though distribution of chromosomes proceeds normally. Consequently, the exact shape of the spindle seems not to be significant in chromosome movement.

2. *Formation of the Anastral Spindle*

The first trace of spindle formation may be observed a considerable time before the breakdown of the nuclear envelope (up to 3–5 hours before BNE in *Haemanthus*), and has the appearance of a clear zone (CZ), usually of uniform thickness, around the nucleus. Sometimes mitochondria are arranged perpendicular to the surface of the nucleus, al-

though it is not known whether they are in close contact with the NE. Later the shape of the CZ changes, due to factors which are not clear. In elongated cells with an ellipsoidal nucleus, the CZ often has a half-moon shape up to the moment of BNE [polar caps described by Robyns; see also Schrader, 1953, and Fig. 1 (see p. 226)]. By contrast, in isodiametrical cells it grows uniformly around the nucleus. In later stages of CZ formation, the polar regions are formed and are seen as streaks of birefringent material (Inoué and Bajer, 1961) which vary in number from one to six (though more than three are rare). Most commonly there are two poles on opposite sides of the nucleus which become the future poles of the spindle; however, if more than two poles are present after the BNE, they usually fuse, change orientation, and the long axis of the spindle occupies an intermediate position (Bajer and Molè-Bajer, 1969). In exceptional cases, more than two poles persist and a multipolar anaphase follows. Multipolarity in the first stages of spindle formation was noticed a long time ago (Wilson, 1928; Schrader, 1953). It is quite apparent from thin sections that, during CZ formation, MTs are rather long (probably several microns) and are irregularly arranged in the form of a meshwork. However, a change in arrangement of MTs and increase of regularity proceeds quickly as prophase advances. MTs tend to be arranged more and more in parallel with the future axis of the spindle and their number increases. It is not clear how these changes of arrangement are achieved. In living cells parts of the CZ can disappear and reappear very easily, and when they reappear they are usually in a different position. This suggests that at this stage MTs are organized and disorganized rather easily. During a disappearance, birefringence diminishes greatly but often does not disappear completely. This may indicate as suggested by Pickett-Heaps (1969a,c), that MTs may break into shorter pieces which rearrange or are transported and grow in the polar directions. In any case it is highly unlikely that considerable movements of completely intact MTs occur at this stage because this would lead to formation of sharp bends and kinks, which are never found.

B. Astral Spindle

1. *Shape*

Asters seen with the LM consist of oriented material radiating from the spindle poles or centrioles. As seen by EM, asters are composed of MTs, membranes, and vesicles, usually as a mixture of these elements; furthermore, the MTs of the asters are morphologically indistinguishable from those in the spindle. Spindles of this type are typical for animals,

but asters are also found in several lower plants during mitosis and in a greater number of organisms in meiosis only. In a few cases, asters have been produced by chemicals, e.g., Piramidon (aminopyrine; Östergren *et al.*, 1953); unfortunately the fine structure of such asters has never been investigated.

2. *Formation of Astral Spindles*

Before the spindle begins to form, the centrioles replicate and their surrounding asters grow slightly. There is then a comparatively rapid separation of asters and a spindle forms between them, usually on one side of the nucleus while the NE is still intact. This classic picture was one of the first described for mitosis, and it stimulated various "repulsion" hypotheses in the nineteenth century.

It is often stated that MTs are formed by centrioles, but so far there is no really convincing evidence that MTs touch the centrioles; rather, MTs end in satellites, or, in exceptional cases at the triplet base (Brinkley and Stubblefield, 1966). These satellites are electron dense bodies with a rather amorphous structure and fuzzy outlines. Although this may not be true for all species or stages, it is likely that satellites are the ends of some growing MTs, i.e., the region where material is added to the tip of the growing MT resulting in its elongation (see Section XII, A,3). During formation of the spindle the MTs between the asters increase in length, forming a thin spindle composed exclusively of continuous MTs; at the same time the asters again increase in size.

Spindle formation has been analyzed *in vitro* in spermatocytes of *Batrachoseps wrighti* by means of time-lapse techniques (Bajer, unpublished). As the spindle forms on the side of the nucleus, the NE seems to break nearly simultaneously in regions adjacent to the asters. At the moment of BNE, the asters usually do not reach completely to opposite poles of the nucleus, but are located far apart and asymmetrically on one side of the NE. After the initial break, the NE fractures along the arch tangential to the outline of the nucleus on the spindle side. These studies have not yet been followed by EM observations, and no data are available concerning such details as behavior of the NE and penetration of MTs.

V. The Role of Centrioles

The premitotic movements of centrioles led to the early conclusion that they are responsible for formation and orientation of the spindle.

Although experimental evidence is uncertain, this view is perpetuated in several textbooks and has been widely accepted. However, it should be stressed that such an interpretation is based mainly on LM observations which were not combined with detailed ultrastructural studies. Very few EM studies have been reported on the structure of the elongating prophase spindle and its relation to the NE. Dietz (1959, 1966, and personal communication) observed spindle formation in mechanically deformed cells during meiosis in the crane fly, and found that even if the asters are displaced as early as late diplotene, the spindle forms in a normal way. Later many of the secondary spermatocytes lack centrioles and asters, but the spindle and chromosome movements are still normal. Dietz concluded that the association of asters and centrioles with the spindle poles simply represents the mechanics of centriole distribution to daughter cells, and that asters are not needed for the formation of either the spindle poles or the whole spindle. Harris (1962a, and personal communication) found that during early development of the sea urchin embryo, asters diminish in consecutive divisions and finally disappear. At the same time the centrioles detach from the spindle and migrate to the cell surface, where they later divide and form cilia (Harris, unpublished observations). Such centrioles are indistinguishable from the basal bodies which are responsible for cilia formation.

Centrioles can disappear and form *de novo* (Dirksen and Crocker, 1966; Sorokin, 1968; Kalnins and Porter, 1969), and they may also migrate long distances (Heist and Mulvanay, 1968). In some instances, a centriole develops during migration, as in mammalian lungs. Here Sorokin (1968) found that the centriole differentiates from an amorphous procentriole, and by the time it reaches the surface of the cell it has the shape and structure of a typical centriole (or basal body). Pickett-Heaps (1968) also suggested that in the alga *Chara* the centriole differentiates from dense amorphous material; in *Chara* the process is similar to that found by Dirksen and Crocker (1966) in rat epithelium, and by Kalnins and Porter (1969) in tracheal epithelial cells of chick embryos.

What then is the role of the centrioles? A clue may be found by comparing meiosis and mitosis in several species. In algae (e.g., *Chara*) and several other plants which form motile gametes, centrioles are present in meiosis but none are found in mitosis (Pickett-Heaps, 1968). It seems that centrioles or blepharoplasts (the latter being multiple centrioles: Mizukami and Gall, 1966), are always present when motility of the cells requires their function. However, in highly specialized systems, variations occur in the division and behavior of centrioles. For example, in honey bee spermatids supernumerary centrioles are formed

(up to 16 in a single primary spermatid). Such centrioles are eliminated by budding and they degenerate; only in exceptional cases do they form flagella (Hoage and Kessel, 1968). In rat epithelium, up to 300 centrioles may form from one initial pair (Dirksen and Crocker, 1966). If these centrioles do not multiply or degenerate, each one produces a single cilium. Consequently, regular distribution of centrioles is very important, and disturbances in centriole distribution lead to abnormalities such as accessory sperm tails (Pollister and Pollister, 1943) or multipolar division (found in experiments with mercaptoethanol; Harris and Mazia, 1962).

From these examples, some speculations may be made:

1. The essential role of centrioles is to produce the motile apparatus necessary for the functioning of a cell.

2. In the course of evolution, the most foolproof mechanism for distribution of centrioles has been developed, involving the location of centrioles at the poles of the spindle and their distribution during anaphase.

3. To make the distribution easier, and to prevent their accidental loss, the centrioles are surrounded by "large" asters which serve the purpose of positioning the centrioles on the spindle poles and facilitating regular distribution.

4. In some tissues, asters may also play a role in determining the planes and normal course of cleavage [see the hypothesis proposed by Costello (1961) for spiral cleavage].

5. Asters in some types of division may play a very important role in chromosome distribution. The presence of the asters may greatly modify chromosome behavior. This very complicated and important problem will be discussed extensively elsewhere (Bajer and Molè-Bajer, 1971).

Some of these conclusions have been reached independently by Dietz (1959, 1966), Nakao *et al.*, (1968), and Pickett-Heaps (1969d). In the latter work, Pickett-Heaps also gives a stimulating analysis of some of the features mentioned above.

VI. Orientation of the Spindle

Discovery of a preprophase band of MTs by Pickett-Heaps and Northcote (1966a,b) seemed initially that it might account for the orientation of the spindle in plants. This preprophase band appears before prophase begins and is composed of long MTs which surround the nucleus and vary in number from a few to 200. Burgess and Northcote (1967) found

such a band in root tips of timothy grass, *Phleum pratense*, and Cronshaw and Esau (1968) found it in leaves of *Nicotiana tabaccum*. Because the preprophase band is located symmetrically around the future equator of the metaphase plate, it was suggested that its function is to align the nucleus prior to prophase; positioning of the nucleus might then determine the orientation of the spindle (Pickett-Heaps and Northcote, 1966a,b; Burgess and Northcote, 1967).

On the other hand, it was recognized that many cells with a well-defined preprophase band do not divide symmetrically. Asymmetric division was studied in detail by Pickett-Heaps (1969a,b,c), especially during development of the stomatal complex in both normal and abnormal divisions. As a result of these studies, Pickett-Heaps (1969a,c) has revised his previous views and states that the preprophase band does not position the nucleus prior to division. For example, he found that the nuclei of *Commelina cyanea* are polarized long before the preprophase band is formed. Consequently, appearance of this band is now regarded as a result rather than a cause of nuclear polarization. According to Pickett-Heaps (1969c), large segments of MTs from preprophase bands are transported into the future spindle; this view is contrary to that expressed by Burgess and Northcote (1967).

A preprophase band is not found in all tissues, e.g., it seems to be absent in several types of root. Furthermore, it could not be found in *Equisetum* (Sakai, 1968), in *Psilotum* (Allen and Bowen, 1966), or in *Haemanthus* endosperm. It is likely, therefore, that more than one factor is responsible for determining the long axis of the spindle. The main influence may be mechanical conditions inside the cell, such as the shapes of the cell and its nucleus, the location of vacuoles in relation to the nucleus, etc. This interpretation is supported by the work of Butterfass (1969), who showed that mechanical conditions are essential in the nonrandom distribution of plastids during late stages of mitosis in guard mother cells of *Trifolium*. Burgess and Northcote (1968) also pointed out that a close relation exists between ER and the aggregation-disaggregation of MTs; they suggested that ER may play a role in the transport of MT subunits. Factors such as the arrangement of chromosomes inside the nucleus, or a nonuniform distribution of nuclear material, may also influence how and where MTs are formed. For example, the volume of the nucleus decreases slightly during prophase, and this process is associated with a change in dry mass (Richards and Bajer, 1961). In endosperm, parts of the chromosomes seem to approach the NE, and long before BNE holes form through which some material passes to the cytoplasm (Bajer and Molè-Bajer, 1969). Although it is not known what substances pass through the NE at this stage, the process somewhat resembles nuclear activity associated with salivary gland

puffs in *Drosophila*. If the material passing into the cytoplasm stimulates formation of the CZ, it might be speculated that the position of chromosomes inside the nucleus could influence the sites where MTs form most extensively, and thereby determine at least partly the long axis of the spindle. As shown by the classic work of Rabl in the nineteenth century (Wilson, 1928), chromosomes inside the prophase nucleus do not reorient; slight chromosomal movements may be due to changes in the shape of the nucleus. Consequently, if the chromosomes are attached to the NE, as suggested by Comings (1968), the sites where MTs form most abundantly in prophase would already be determined during the previous telophase.

One more property of MTs should be mentioned: MTs are often arranged parallel to one another and in bundles. However, it is not known to what extent they assemble in bundles as opposed to arranging themselves in bundles afterward, i.e., whether their orientation is mutually dependent.° The appearance, role, and origin of MT doublets, found most clearly in the interzonal region, deserves special attention (Stubblefield and Brinkley, 1968; Robbins and Gonatas, 1964; Brinkley *et al.*, 1969). Whatever the process resulting in mutual orientation of MTs, EM observations show that just before the NE breakdown, most MTs are arranged parallel to the long axis of the future spindle.

VII. Breakdown of the Nuclear Envelope (BNE)

The fine structure of the NE is basically the same in plants and animals; it consists of two membranes (totaling 450–600 Å wide) into which are set nuclear pore complexes, each of which has eight subunits (Franke, 1970). The process of BNE has been studied at the fine structural level in *Haemanthus* endosperm by Bajer and Molè-Bajer (1969); in other species few data concerning this process are available, but it is probable that the general scheme is similar (with some modifications). Pieces of the NE persist throughout mitosis in root tips of *Allium* (Porter and Machado, 1960). In leaves of *Nicotiana tabaccum* (Esau and Gill, 1969a), and in some animals like the newt (Davies and Tooze, 1966), the pore complexes may disappear in these fragments shortly after NEB. On the other hand, pore complexes may persist for a long time in *Haemanthus* endosperm. Usually the BNE requires about 3 minutes in *Haemanthus* (Bajer and Molè-Bajer, 1969), and 2 minutes in *Chaetopterus* eggs (Merriam, 1961). The NE breaks into several

° *Added in proof:* Recently evidence has been obtained that long MTs are capable of transverse movement in the phragmoplast (Bajer and Molè-Bajer, 1971).

pieces or pinches off in the form of small vesicles which are indistinguishable from other types of vesicles; the latter can be found in *Chaetopterus* eggs. In some species, BNE may last longer, e.g., in mesophyll of *Nicotiana tabaccum* (Esau and Gill, 1969a).

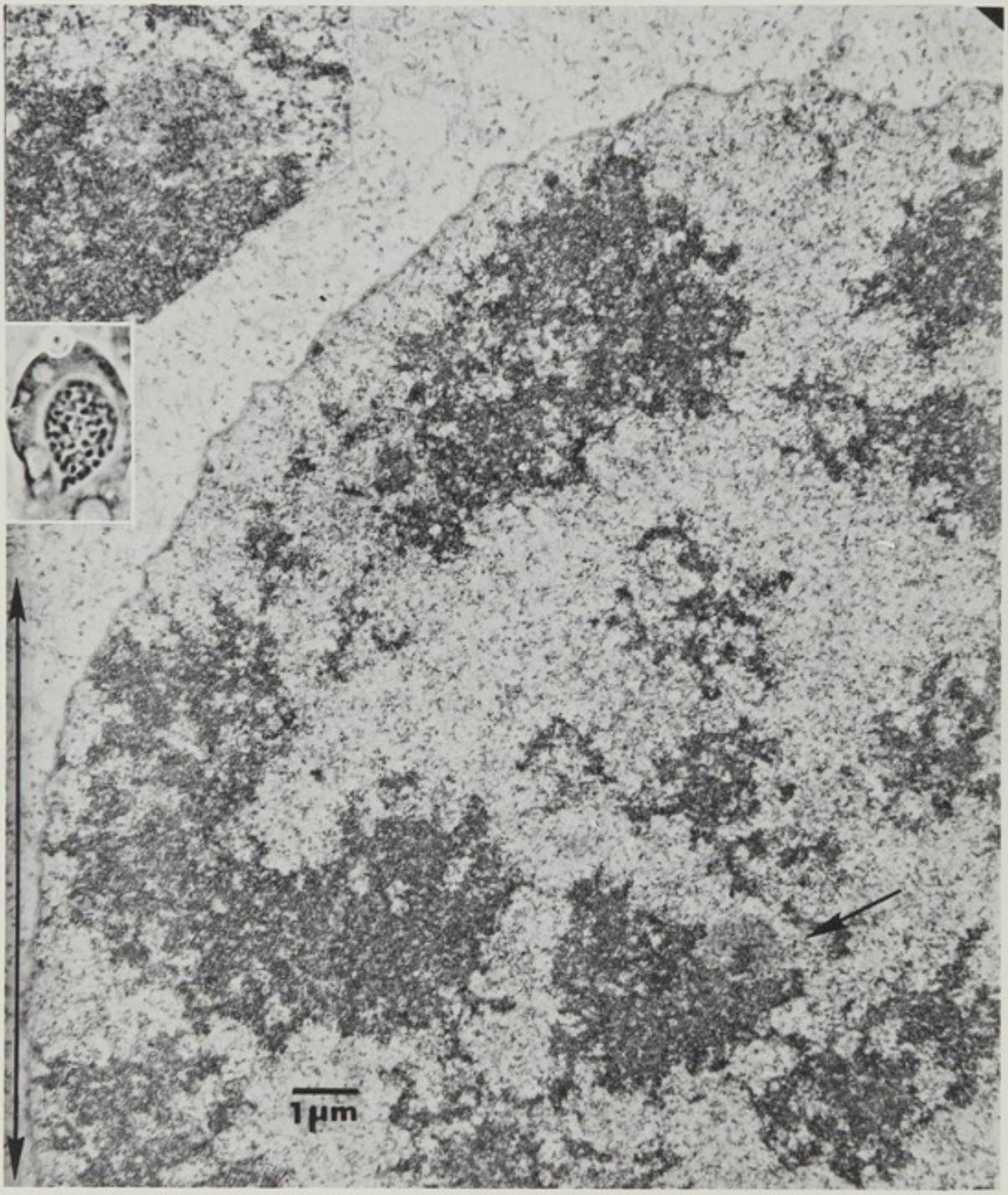


FIG. 1. Prophase nucleus of *Haemanthus katherinae* endosperm 1–2 hours before BNE. The double pointed arrow indicates the predicted long axis of the spindle. Several protrusions of the NE are seen, especially in the polar region (cf. also Bajer and Molè-Bajer, 1969). The interior of the nucleus is devoid of MTs. The denser core in a kinetochore is clearly visible. *Inset:* LM photomicrograph of the same cell embedded in plastic.

Undoubtedly, changes occur in the NE during prophase. In *Amoeba proteus*, these changes seem to be generated by the nucleus and not by the cytoplasm (Feldherr, 1965). Hancock and Ryser (1967) suggested that a histone fraction may be released from the DNA during prophase which structurally changes (weakens) the NE. However, the

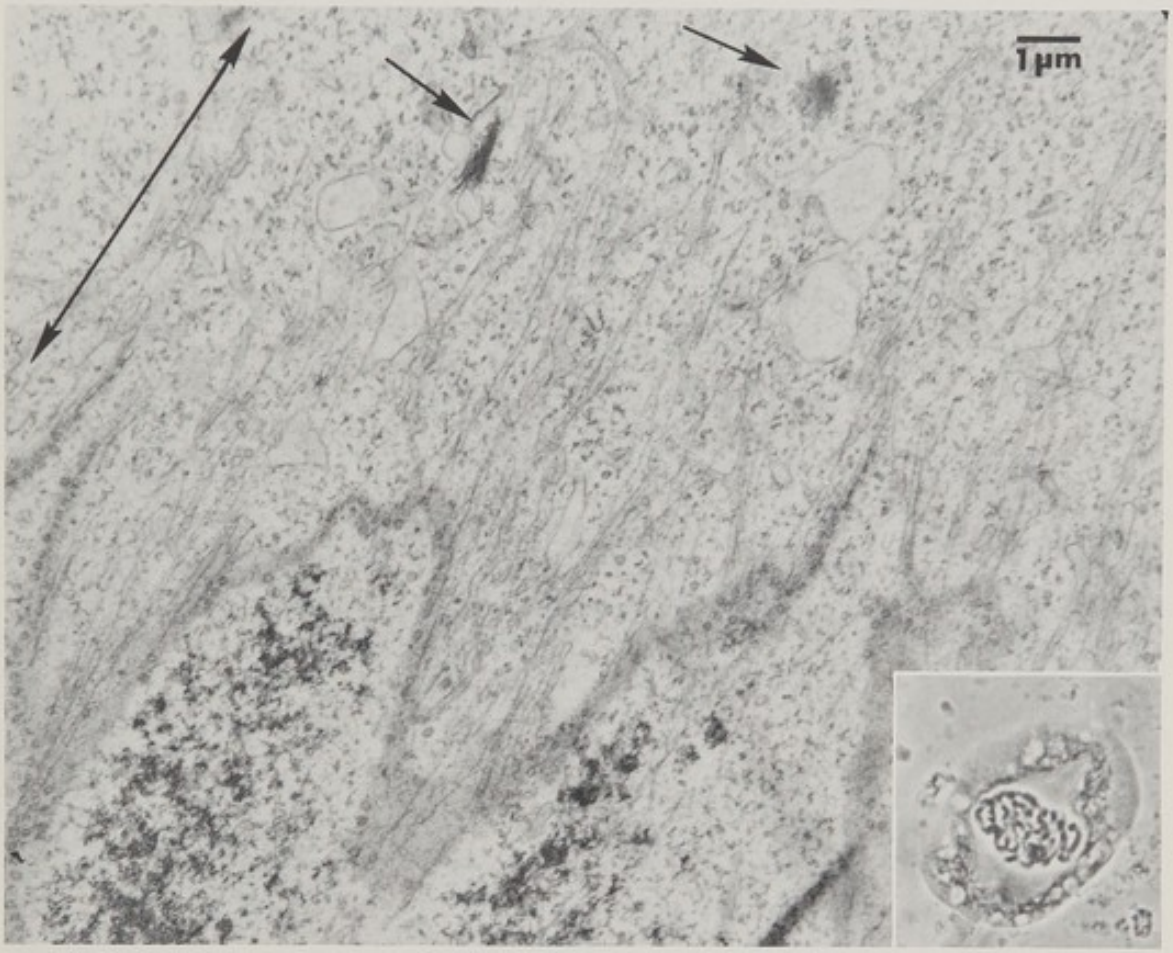


FIG. 2. Penetration of MTs during BNE in *Haemanthus* endosperm. The double pointed arrow indicates the predicted future long axis of the spindle. Tangential sectioned MTs from the CZ penetrate the nucleus as irregular bundles and cause invaginations of the NE. The ER is arranged mostly parallel to MTs. The two short arrows show GBs. *Inset:* LM photomicrograph of the same cell embedded in plastic.

actual break seems to result from the mechanical pulling and pushing of the NE by the MTs, which begins long before BNE (Fig. 1) and intensifies as the cell approaches the moment of breakdown. In *Haemanthus*, as a rule, MTs are attached to the NE in the polar regions by means of lateral connections, but never by the ends (Bajer and Molé-Bajer, 1969); such MTs seem to pull the NE, but others exert some pushing and finally penetrate into the nucleus in bundles of a few or many MTs (Figs. 2 and 3). MTs from one pole may even approach

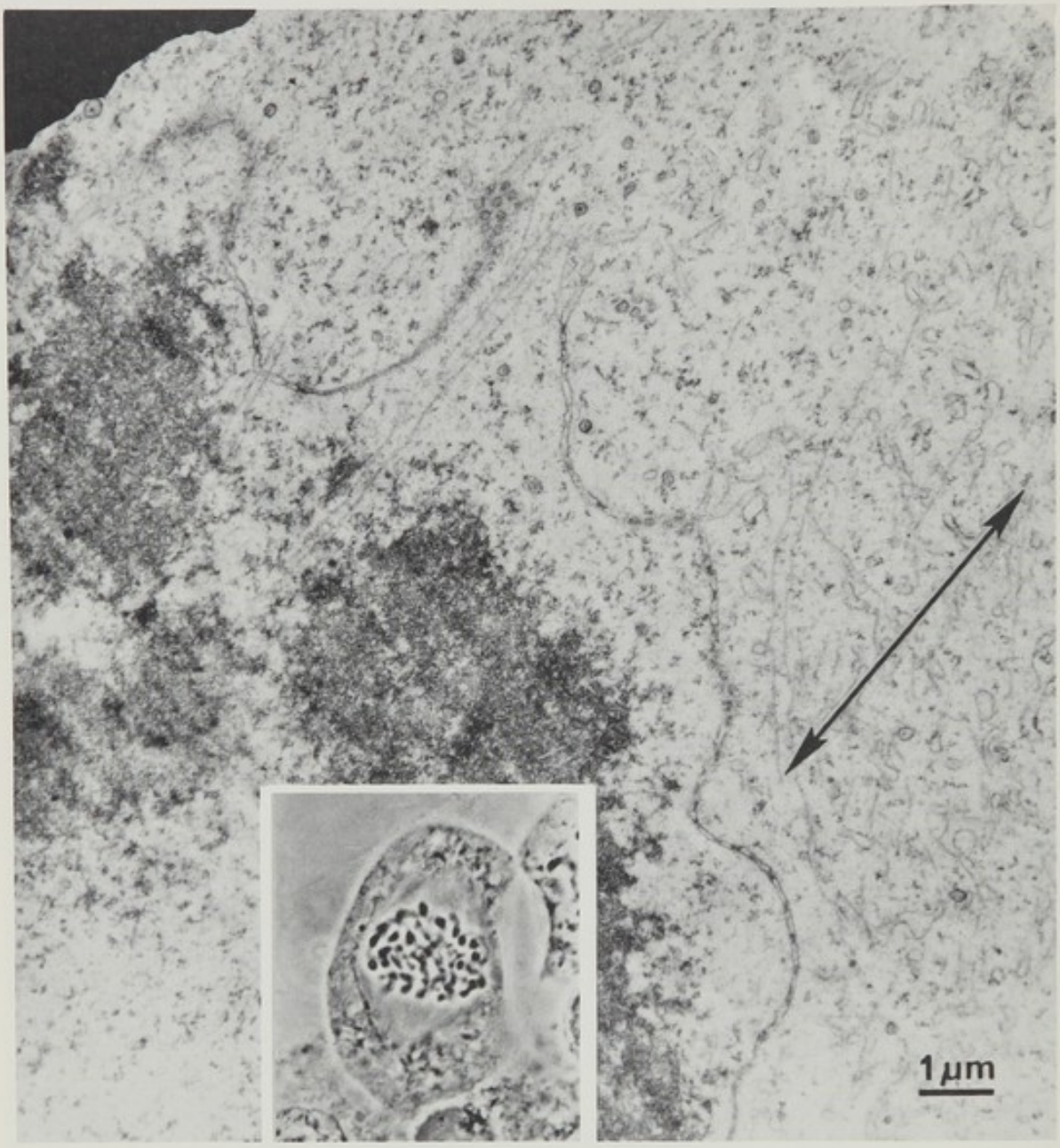


FIG. 3. BNE in *Haemanthus* endosperm. The double pointed arrow indicates the predicted future long axis of the spindle. MTs from the CZ penetrate through the ruptured openings of the NE. *Inset:* LM photomicrograph of the same cell embedded in plastic.

the vicinity of the opposite pole. Reports that MTs pass through the nuclear pore complexes and attach to the chromosomes in late prophase (Ledbetter, 1967) have so far not been confirmed; however, MTs may pierce small fragments of the broken NE in later stages of anaphase (Robbins and Gonatas, 1964).

In *Haemanthus,* pieces of the NE often persist to the next division and are not incorporated into the daughter nuclei or resorbed in the

cytoplasm; such pieces may retain their typical structure including nuclear pores. More usually the pieces of broken NE are used in the re-formation of a new envelope at telophase. It is not known whether the NE can form *de novo* from ER. In some cases, the pieces can be followed in LM from prophase to telophase, but unless time-lapse techniques are used it is not possible to distinguish such pieces from the tonoplast (vacuolar membrane). The occasional presence of pieces of NE led Wada (1966) to an incorrect conclusion concerning the intranuclear origin of the spindle; he stated that the NE persists throughout division and does not fragment into pieces. In higher plants and animals these fragments tend to diminish in size and disappear.

VIII. The Structure of Kinetochores

In most species of higher plants the fine structure of kinetochores seems to be similar, consisting of a poorly defined diffuse ball with a more electron dense core (Figs. 1 and 9–12; see also Dietrich, 1966). Such balls have not been seen in the LM, despite the fact that the size is large enough to be noticed (0.4–0.6 μ in *Haemanthus*). The core may have protrusions in cross section, which sometimes form a more organized structure (70–100 Å fibrils combined with 250 Å fibers; such a structure has been reported in *Lilium* by Braselton and Bowen, 1969). In *Haemanthus* the kinetochores are distinguishable long before the NE breaks up and they remain basically unchanged until telophase. MTs attached to the kinetochore most likely are embedded in the electron lighter material; some of them seem to end as inverted funnel-shaped structures or are conspicuously embedded in less electron dense material (Bajer, 1968c). In lower plants (e.g., the alga *Oedogonium*) several distinct layers can be seen in thin cross sections, which resemble the structure of kinetochores in animals (Pickett-Heaps and Fowke, 1969).

The fine structure of kinetochores in higher animals, such as rats or the Chinese hamster, is different from that in higher plants (Brinkley and Stubblefield, 1966; Jokelainen, 1967). In prometaphase and telophase the kinetochore is composed of at least three zones: an inner zone adjacent to the chromosome body; an outer, denser zone; and a lighter zone between. The outer, denser zone often has lighter, diffused material facing the spindle. MTs are embedded in the outer layer or in all three layers. The outer layer is interpreted by Brinkley and Stubblefield (1966) as being composed of a pair of axial fibrils 50–80 Å in diameter,

which are coiled together and which form loops in the lighter zone. On the other hand, Luykx (1970) interprets the same structure as composed of a series of discs. No clear segmentation of kinetochore into three zones has been found in lower animals such as the cockroaches *Blatoides germanica* and *Periplaneta americana* (Krisham and Buck, 1965), or in sea urchins (Harris, 1962a,b).

Since kinetochores have a rather characteristic structure, they should have been noticed in EM studies of the interphase nucleus if their organization remains unchanged. Since they have not been found during interphase, however, it is probable that they must develop as morphological units during early stages of chromosome differentiation under the action of certain genes (Luykx, 1970). Such a speculation is especially attractive because it would explain several disturbances resulting from delay or nonfunction of kinetochores, e.g., chromosomes lagging in anaphase, chromosome elimination in prometaphase, and some aspects of the behavior of univalents. Some support for the concept that certain types of kinetochore malfunction may be due to genetic factors is obtained from genetic studies; in interspecies hybrids between grasses, the two sets of chromosomes often behave independently during meiosis: one set may form only univalents which move toward the metaphase plate and are distributed at a different time from the bivalents (Bleier, 1930; Wagenaar, 1961). Variability and a synchronous behavior by sex chromosomes may also be caused by such genetic factors.

Diffuse (holocentric) kinetochores most likely have a somewhat different structure. In species with such kinetochores the chromosome movements are very characteristic: i.e., the chromosomes are not bent in anaphase but move apart in parallel. Furthermore, after fragmentation by ionizing radiation, the fragments are not eliminated but are distributed like normal chromosomes. Such diffuse kinetochores are found in various groups of plants and animals, including *Luzula, Scorpion* (in the latter it was initially reported as a double kinetochore which has not been confirmed), several insects, and some algae, e.g., *Spirogyra.* An investigation of fine structure in the diffuse kinetochore of *Rhodnius* revealed extensive laminar structures to which the MTs were connected along the entire length of the chromosomes (Buck, 1967).

Several important LM studies established preliminary data about kinetochore structure and function (Schrader, 1953; Lima-de-Faria, 1958), but only a few corresponding observations have been confirmed with the EM. In some species with large chromosomes, kinetochores can be followed in living cells. In *Haemanthus,* the kinetochores are already double at prophase and a hole is seen between the sister kinetochores both in the LM and the EM. In late metaphase the two sister

chromosomes (chromatids) are held together only in the regions on either side of the kinetochores (proximal regions of the chromosomes), most probably by specialized chromomeres. In exceptional cases seen in time-lapse films of *Haemanthus*, one connection breaks but the second one is sufficient to hold the two chromatids together; the chromosomes are then distributed normally. The kinetochore region can be stretched considerably, especially during prometaphase and early metaphase in the first division of spermatocytes, and also in plant mitosis. This stretching often ceases by the beginning of anaphase, a fact which led Dietz (1956) to suggest a change of force during mitosis. The kinetochores can be stretched by several microns, and at the end of the stretched region a small granule (or two granules) have been seen in fixed preparations (Schrader, 1953). Two granules have also been observed in unstretched chromosomes (Lima-de-Faria, 1958). However, combined LM and EM observations on a variety of animal chromosomes in tissue culture undertaken by Hoskins (1969), especially to elucidate these structures, failed to confirm the existence of such granules at the fine structural level. The stretching process is of short duration and unless cells are preselected with the LM for subsequent studies with the EM the chance of finding such events with the EM are slight (see Jokelainen, 1967).

IX. Chromosome Movements

Several aspects of chromosome movement in plants have been reviewed by the authors previously, and only a short summary will be given here (Bajer and Molè-Bajer, 1963; Bajer, 1966). The basic features of chromosome movements in higher plants are presented in Fig. 4. Although details of division may vary in different species, the essential scheme of behavior is the same.

A very important feature, often overlooked, is that the movements within the spindle during prometaphase, and again within the phragmoplast during telophase, occur in opposite directions: i.e., the chromosomes move toward the equatorial plate while bodies unattached to the spindle move toward the poles. This tendency to eliminate bodies out of the spindle is important in both plant and animal mitosis, but the process is better understood in plants. Some data on the behavior of fragments in fixed animal material were obtained by Carlson (1938), who found that in the neuroblasts of *Chortophaga viridifesiata* fragments without kinetochores were eliminated poleward during anaphase, and

often were included in the sister nuclei. However, some fragments stayed close to the metaphase plate. Carlson's description suggests that the fragments detach at the start of anaphase, as they do in plants. It seems likely that the fragments studied by Carlson were connected to metaphase chromosomes until the start of anaphase. In general, chromosome fragments, small granules, and nucleoli, are eliminated at a speed which is independent of size and which is the same or occasionally slightly greater than the speed of the chromosomes during anaphase. Because of this tendency for elimination, other cell organelles do not enter the

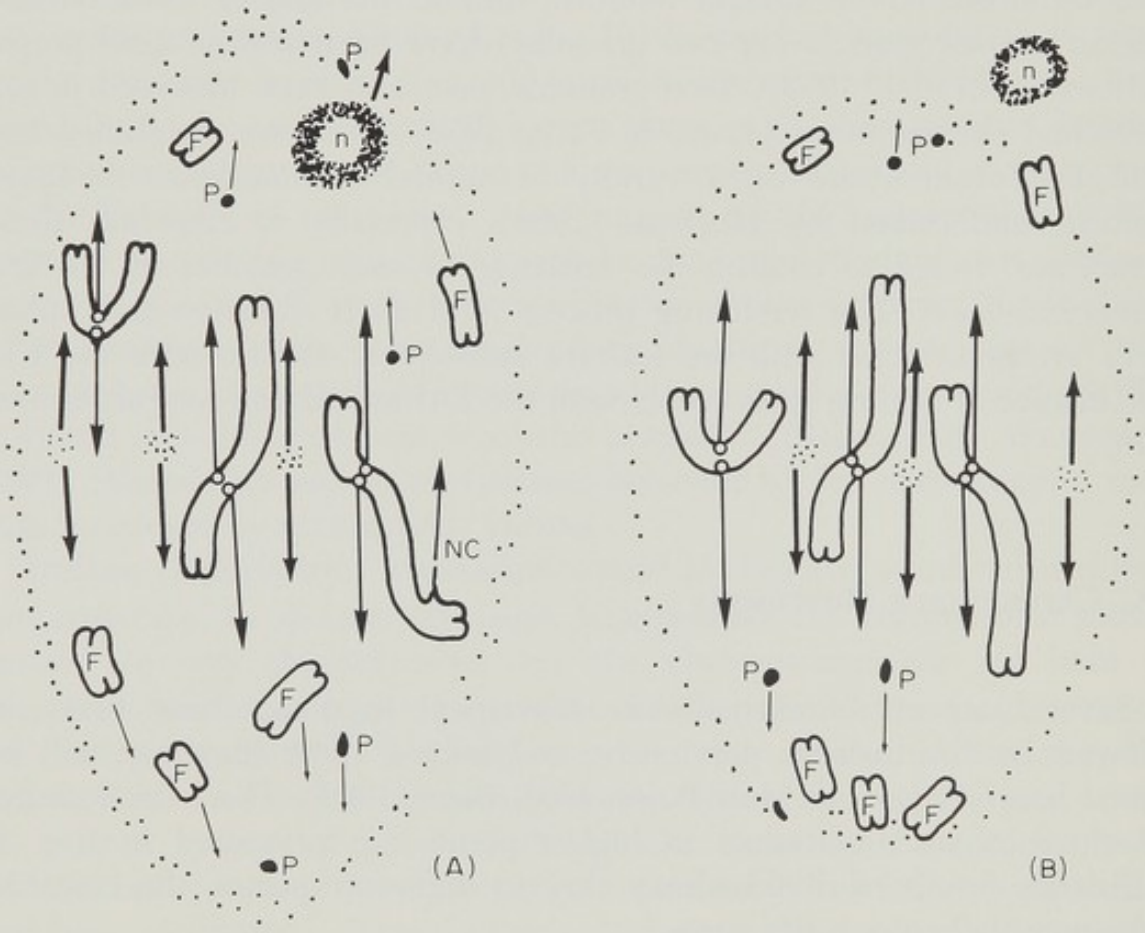


FIG. 4. Schematic representation of movements and directions of forces acting in the spindle and phragmoplast during plant mitosis. White circles represent kinetochores; N, persisting nucleolus; F, acentric fragment; P, small particles (some of them are dictyosomes). Mitochondria and small particles accumulate on the border between the cytoplasm and the spindle. Small dots and thick arrows indicate the movements of particles (some of which may be dictyosomes), as observed with the Nomarski system (Allen *et al.*, 1969). (A) During prometaphase bodies unattached to the spindle are transported toward the poles, and some chromosome arms execute neocentric movements (NC). Kinetochores tend to be arranged on an equatorial plane. (B) At metaphase most of the acentric bodies have been eliminated at the spindle poles, and the chromosome arms are arranged parallel to the long axis of the spindle.

spindle, or are not abundant there. Thus, in virus-infected plants Esau and Gill (1969b) found viruses in the spindle only very rarely, although large virus inclusions were often seen around the spindle or the nucleus. A few mitochondria and dictyosomes are often present inside the spindle. Smaller particles (e.g., vesicles produced by dictyosomes) seem to be transported in both directions, because they are uniformly distributed throughout the spindle, especially in later stages.

During prometaphase, the kinetochores may move to the pole or poles

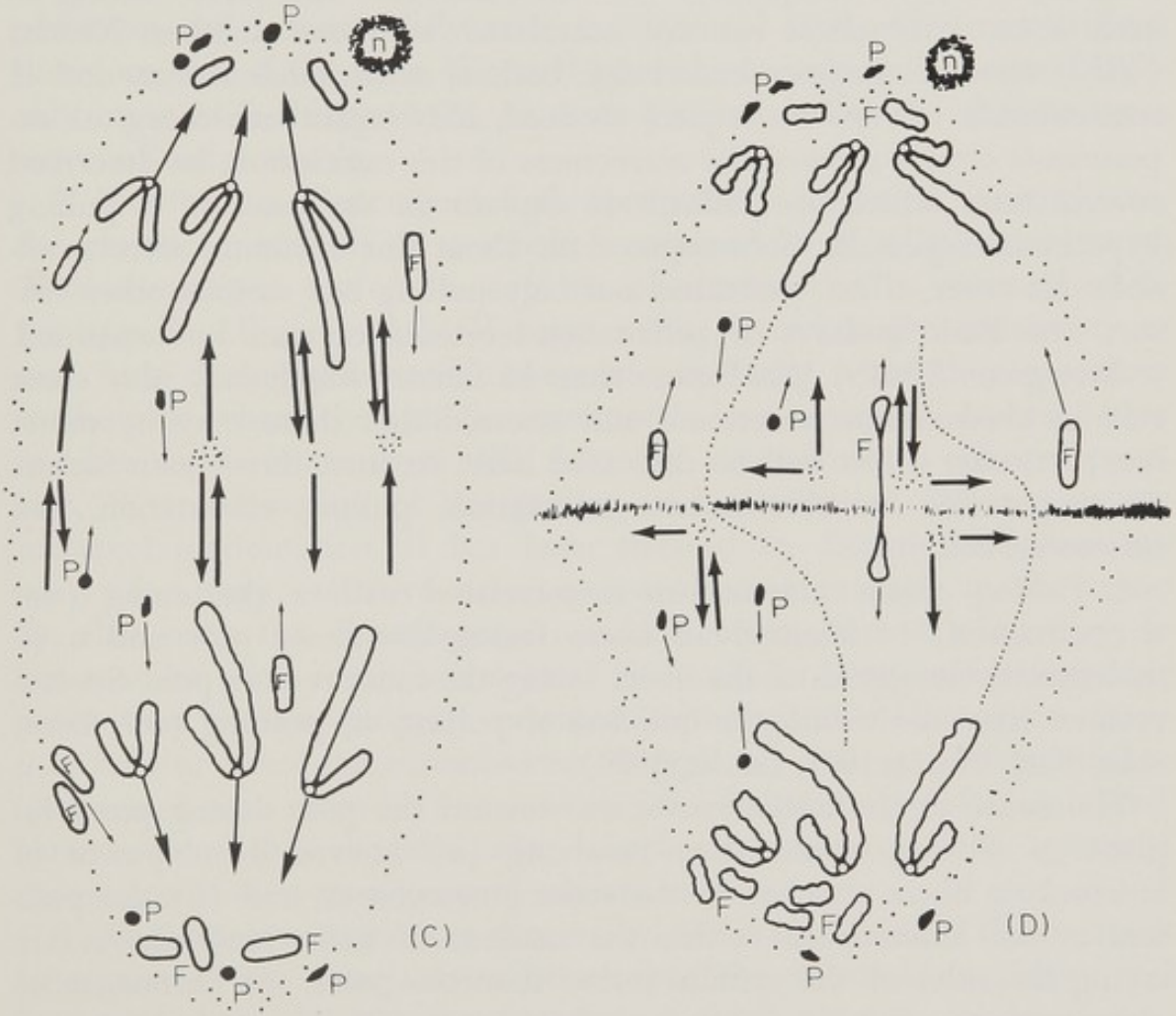


FIG. 4 (*cont'd*). (C) At anaphase, daughter kinetochores move toward the opposite poles and some acentric bodies in the interzonal region (fragments, particles) move toward the equator. The spindle has elongated. (D) During telophase the lateral movements appear within the phragmoplast, when the kinetochores and a majority of acentric fragments are at the poles. Fragments which moved toward the plate now move to the poles, and those which are caught by the cell plate are torn apart. Lateral movements are clearly seen (e.g., the paths of long trailing chromosome arms which do not follow the path of kinetochores), and they persist for a long time at the edges of the forming phragmoplast. The general poleward tendency of transport prevents movement of some particles and dictyosomes toward the plate.

several times before the beginning of anaphase. The initial movement toward the pole often is caused by erroneous orientation of a kinetochore. However, repeated movements between the poles are especially clear in meiosis, both in animals and plants, as well as during mitosis in most animal cells. The movements give the impression that they result from unbalanced forces pulling the sister kinetochores toward opposite poles. Seto *et al.* (1969) have found that the movements of sister kinetochores in salamander spermatocytes vary independently both in magnitude and in frequency; this indicates that the forces acting at each sister kinetochore are not correlated with one another. Nicklas (1967) stressed that our knowledge both of normal orientation and of reorientation is very inadequate. Indeed, his elegant microsurgical experiments clearly showed the correctness of this conclusion; he described several facts which are difficult to explain on the basis of a pulling hypothesis, especially because no data about fine structure were available. However, if one considers not only pulling but various other factors, the basic features of orientation-reorientation can be explained.

Östergren (1949b) listed more than 15 factors which may play some role in chromosome movement and stressed that there may be more; however, only a few will be discussed here. At least three main factors are responsible for chromosome orientation: pulling, elimination, and neocentric activity.

1. *Pulling* of the kinetochore is correlated with a shortening (*not* a contraction) of kinetochore fibers facing the closer pole and a simultaneous elongation of the fibers facing the more remote pole. Several reviews have dealt with the question of pulling vs. pushing (see Cornman, 1944; Mazia, 1961; Luykx, 1970).

The cause of the initial movement toward the pole during prometaphase is often a combination involving (*a*) uneven development of kinetochore fibers attached to the sister kinetochores, and (*b*) the position of the kinetochores within the nucleus. If sister kinetochores are facing the sides of the spindle rather than the poles, the chromosomes often move to one pole. It has been found that MTs of such misoriented kinetochores are connected to one pole only (Bajer and Molè-Bajer, 1969), and the effect is the same as if MTs were not developed at one kinetochore in normally oriented chromosomes (i.e., sister kinetochores facing opposite poles). Misoriented chromosomes moving to one pole are usually quickly reoriented, assume normal positions, and move toward the equatorial plate. The fact that the fibers of two sister kinetochores can develop differently is evident from the work of Brinkley *et al.* (1967a), who showed that after the action of colcemid in weak concentrations only the fibers facing the centriole remain. An analysis

of chromosome movements during abnormal monopolar meiosis in the salamander *in vitro* also indicates that the kinetochore fibers of sister kinetochores may develop unevenly (Seto *et al.*, 1969; see also Bělař and Huth, 1933). In *Haemanthus* kinetochore fibers often develop differently on sister kinetochores at the start of prometaphase; this is clearly seen after the action of chloral hydrate (Molè-Bajer, 1967, 1969).

Extensive evidence that orientation and co-orientation are due to pulling was provided by Östergren (1951); this interpretation, in spite of some criticism (cf. Nicklas, 1967), still offers a rather complete explanation and is most likely correct in general terms. We may assume, therefore, that if the fibers directed toward one pole are stronger (probably more MTs, longer MTs, or more "active" MTs) the chromosomes move toward that pole.

2. *Elimination.* Concurrent with the pulling action on kinetochores, the chromosome body as a whole is exposed to an elimination force. Such elimination forces are capable of rotating bodies unattached to the spindle (Bajer and Molè-Bajer, 1963). Accordingly, chromosomes may turn during poleward movement, especially when they are close to the pole where the spindle structure is less organized, having fewer and less regularly arranged MTs. After the chromosome is oriented properly, the combination of the pulling and elimination forces prevents incorrect reorientation. It has been stressed by Dietz (1969), on the basis of a very extensive time-lapse analysis of meiosis in the crane fly, that once the chromosomes reach an orientation stability they do not reorient. As a result, when sister kinetochores begin to face opposite poles, they remain properly oriented. Although the elimination force may help to orient the chromosomes, it probably acts as a safety mechanism which may often be unnecessary for a reorientation of misoriented chromosomes. It is doubtful, also, whether it can secondarily misorient chromosomes once they are properly oriented.

3. *Neocentric activity* is a process in which the spindle fibers attach to a part of the chromosome other than the kinetochore (Bajer and Östergren, 1961). It can be regarded as another safety mechanism or as a vestige of kinetochore activity. The process was first described in meiosis of corn by Rhoades and Vilkomerson (1942) and is in *Haemanthus* of short duration: 1–2 minutes but, exceptionally, 10 minutes or more. Neocentric activity is especially pronounced during meiosis, i.e., when orientation of kinetochores is especially important; during mitosis neocentric activity is more often seen in the early stages of division (prometaphase) and is only rarely observed during anaphase. Its peak intensity evidently coincides with the time when any misoriented chromosomes would have to be reoriented if they are to be regularly

distributed at anaphase. Especially during the early stages of prometaphase, MTs from one kinetochore may engage both poles (Luykx, 1965a,b), or MTs may be seen between two nonsister kinetochores. Such misconnections usually break up during the later stages of prometaphase-metaphase. However, analysis of chromosome orientation and velocity indicates that such misconnections may occasionally persist throughout division (Bajer and Östergren, unpublished observations). This is also seen during some types of nondisjunction when the chromosome executing nondisjunction moves more slowly than others at anaphase.

In summary, the metaphase position of kinetochores is probably achieved as a result of several factors (Östergren, 1949a,b, 1950): most likely pulling and elimination are the main ones. As a result of the interaction among these factors, kinetochores at the start of anaphase occupy approximately a mid-position in the spindle, and the chromosome arms either tend to be arranged parallel to the long axis of the spindle (as in most plants with large chromosomes) or else are eliminated outside the spindle (as in the hollow spindle of the newt). A striking difference in metaphase of plant and animal cells is that chromosomes in plants are nearly motionless, while in animal cells they often oscillate continually until the start of anaphase. However, even in animal cells the oscillation decreases during metaphase (Dietz, 1969). Dietz suggested that this is due to a decrease of the pulling force during metaphase. Asters if developed may considerably modify the behavior of chromosomes. The problem is discussed elsewhere (Bajer and Molè-Bajer, 1971). Although the direction of force within the asters has not been analyzed in detail, time lapse movies of newt cells (cf. Östergren *et al.* 1960) indicate that movement occurs in both directions (i.e., small bodies move inside the aster and large bodies move outside).

A related problem is the question of what triggers the start of anaphase. Pull alone is not sufficient to explain the beginning of anaphase movement. Both in animals (Carlson, 1938) and in plants (Bajer, 1966) acentric fragments split at the same time that kinetochores separate, indicating that both extra- and intrachromosomal triggers are involved. A frame-by-frame analysis of the start of anaphase in *Haemanthus* endosperm shows that this event is not precisely simultaneous for all chromosomes. However, the difference in time is so small that it is not noticed during film projection. It has been speculated that changes in chromosome structure, such as delayed replication in the regions adjacent to kinetochores, lead to the breaking of the bond holding two sister chromatids together. Detailed observations suggest that anaphase separation in one chromosome is enough to trigger this event in all chromosomes.

The phenomenon is seen best in cells with many chromosomes, where the chromosomes often begin anaphase at one locus and the movement spreads progressively; in this way the "anaphase condition" seems to be transmitted from one chromosome to another. This may explain why even fragments lying in the cytoplasm split at the same time, or with slight delay, when the chromosomes start anaphase. Another observation indicating a possible biochemical trigger for anaphase is that during metaphase the spindle shortens in most cells; although the significance of this process is not clear, it can be speculated that elimination of some blocking material is necessary for anaphase to start. This would explain why metaphase in unhealthy cells is prolonged and anaphase may never occur. The transport activities of such abnormal spindles are very weak (see Section I).

In a great majority of cells two processes occur during anaphase; elongation of the spindle and shortening of the half spindles. There is great variation in the magnitude of these two processes. Since an extensive review of these variations was provided by Mazia (1961), the anaphase events will not be discussed here.

X. Telophase and Cytokinesis

Both in plants and animals cytokinesis is a process separate from mitosis. Nevertheless, it is usually (but not always) triggered by mitosis or meiosis. During division of a syncytium such as endosperm or insect blastoderm, cell walls may form between sister nuclei long after mitosis. This phenomenon was first described by Scheiden and Schwann in the nineteenth century and was studied in detail by Jungers (1931). In animals, cytokinesis can occur in cells which completely lack nuclei (Schrader, 1953; Wilson, 1928). Preparations for cytokinesis begin during anaphase and not, as is often stated, in telophase. Consequently, the beginning of cytokinesis is superimposed on the final stages of chromosome distribution, as the chromosomes are still moving to the poles. Changes involved in the initial stages of cytokinesis actually influence the chromosome movements and may be partially responsible for the behavior of chromosomes during late or middle anaphase.

There are some similarities between the formation of a phragmoplast and of a mid-body, although these two structures seem very different, the former being found only in plants while the latter as a rule occurs only in animals and some lower organisms. The mid-body begins to form in mid-anaphase, and after mitosis is completed it develops as

an equatorial constriction of the remaining fibrous spindle structure. Consequently, it has the form of two diverging cones fused at their apices. Similarly, the phragmoplast also begins to form in anaphase, and when fully developed at telophase it is a fibrous, usually barrel-shaped structure lying between sister nuclei; it is responsible for the formation of the cell plate. Activity by MTs in both the stem body and phragmoplast is well documented. In both cases this activity is evident in the formation of "waves" resembling peristaltic movements which seem to be associated with activity of straight MTs; such waves have been reported during post-telophase activity of the stem body in HeLa cells (Byers and Abramson, 1968) and in the phragmoplast as described by Bajer and Molè-Bajer (1963), then later analyzed more precisely by Allen, Bajer, and LaFountain (1969).

A. Plant Cells

Transport properties within the phragmoplast are similar to those in the prometaphase spindle, i.e., transport occurs in two opposite directions (Fig. 4): Material responsible for cell plate formation is transported toward the equatorial plate, while at the same time acentric fragments are transported toward the poles (Bajer and Molè-Bajer, 1963). The phragmoplast also prevents sister nuclei from approaching one another; thus, the collapse of the phragmoplast at an early stage of its formation, e.g., in mid or late anaphase before a cell plate appears, often leads to formation of a restitution nucleus with two sets of sister chromosomes and double chromosome number. Such a collapse of phragmoplast structure occasionally occurs spontaneously (Fig. 5), or it can be produced by a variety of experimental factors such as colchicine, heat, or cold; the event is especially dramatic when seen in time-lapse films.

The phragmoplast is also responsible for the lateral movements of chromosome arms and the movement of fragments toward the equator, which is occasionally observed. During lateral or transverse movements the long trailing chromosome arms do not follow precisely the paths of the kinetochores but swing toward one side of the phragmoplast (Bajer and Östergren, 1963). Analysis of the behavior of phragmoplast fibrils, composed of a small number of MTs (Bajer and Jensen, 1969), indicates that the fibrils bend (Fig. 6) and mechanically push the chromosome arms toward the edge. Lateral movements are not seen clearly in all endosperm cells, but they are always seen in flattened cells or short cells; it is most likely that they can appear in any plant cell which

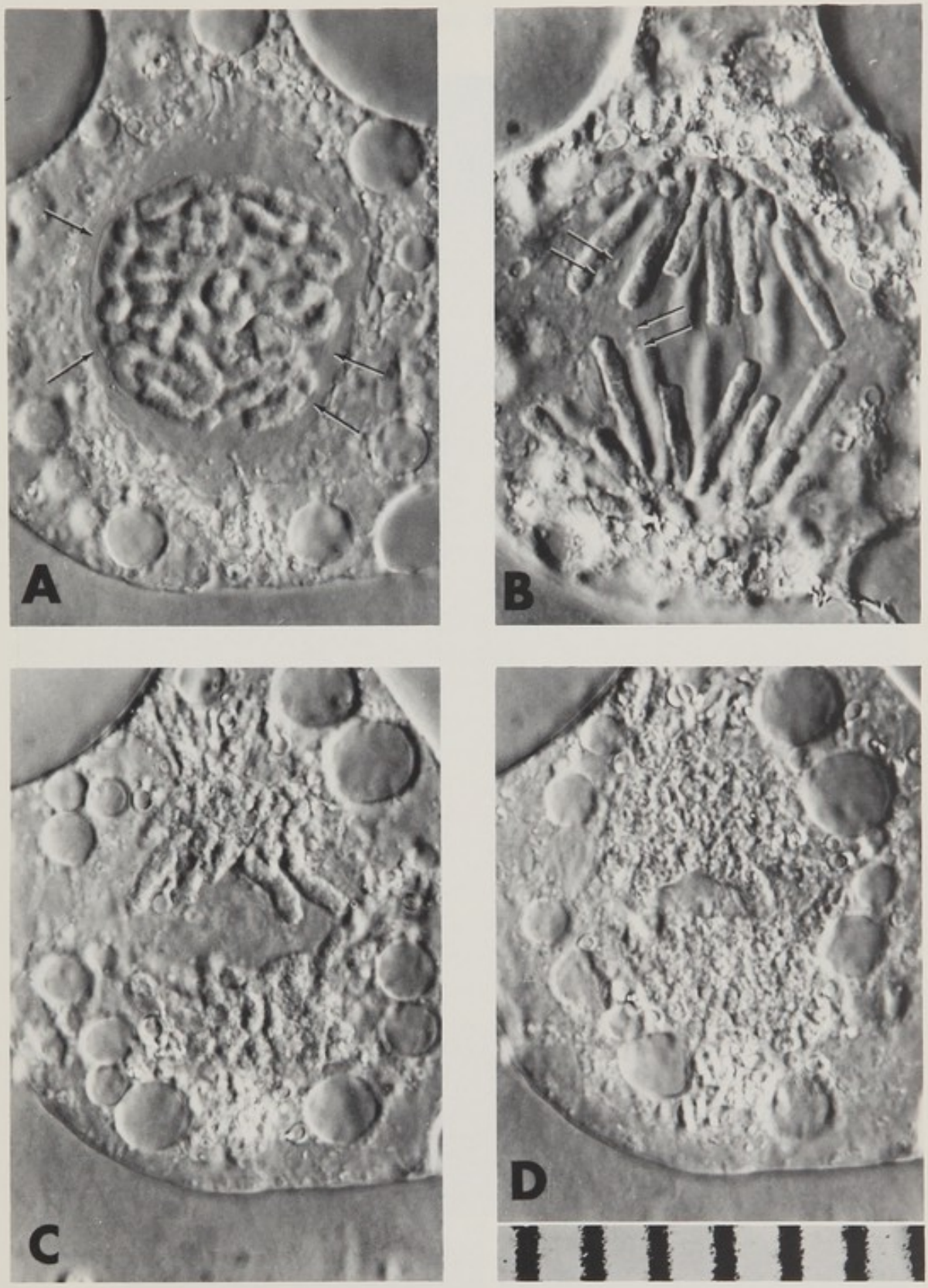
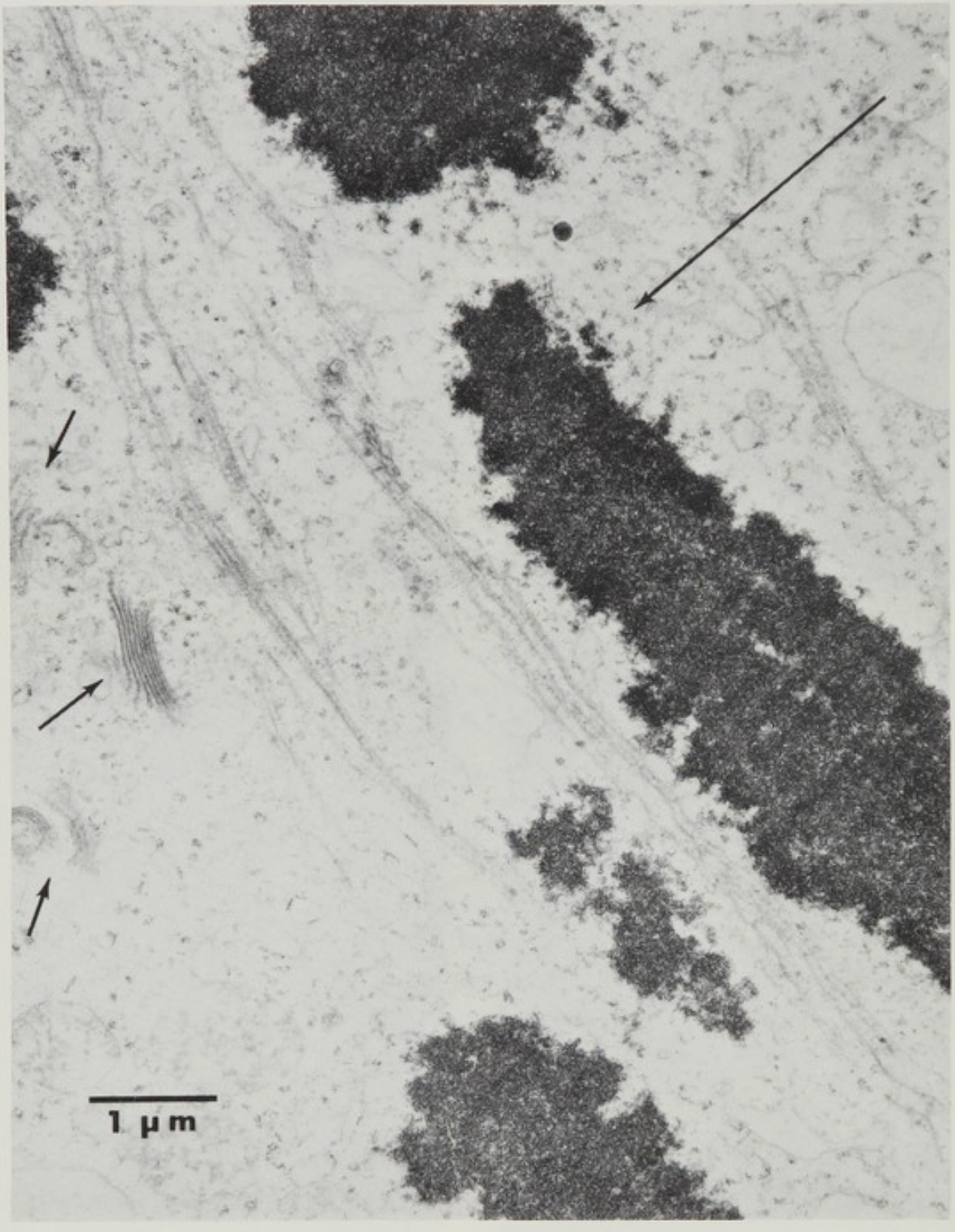


FIG. 5. The effects of failure of phragmoplast formation on chromosome distribution in *Haemanthus katherinae* endosperm. (A) Start of prometaphase (0 hr). NE broken at the poles but remnants are seen on both sides of the spindle (arrows). (B) Late anaphase (2 hr, 28 min). The position of arms indicates lateral movements which occur in the initial stages of phragmoplast formation. Dictyosomes (arrows) are present in the interzonal region. (C) The phragmoplast fails to form for unknown reasons (3 hr, 29 min). (D) Sister nuclei are nearly fused (5 hr, 4 min). Nomarski interference contrast. Scale intervals in D = 10 μ.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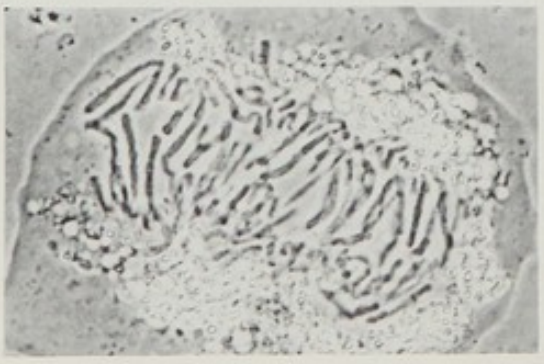

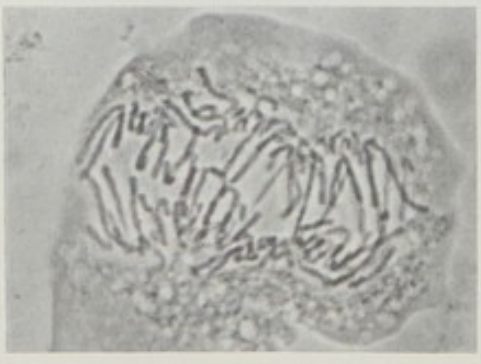

forms a phragmoplast. Continuation of this process is responsible for the sideways growth of the phragmoplast. The most spectacular growth of the cell plate is observed in large vacuolated cells, where the phragmoplast grows laterally through a thin layer of cytoplasm a long distance beyond the primary spindle (Sinnott and Bloch, 1941). In *Haemanthus* endosperm, the cell plate can grow across large vacuoles and MTs visibly push the newly formed phragmoplast sideways.

In early stages of phragmoplast development some acentric fragments occasionally move toward the equator. Such movements by fragments and by univalents in meiosis (e.g., in some grass hybrids) are difficult to explain unless detailed analysis of their behavior is made; the movements of univalents can probably occur in a variety of ways, including delayed activity by the kinetochore. However, as a rule they remain at the equatorial plate during anaphase when other chromosomes move toward the poles. However, in some cells they may return toward the equator at a later stage. Judging from several drawings by Bleier (1930), some univalents move toward the plate in mid-anaphase; thus, the behavior of such acentric fragments resembles the behavior of univalents. In *Haemanthus* if the movement of fragments toward the equator occurs at all, there are only 5–10 minutes within the 25–45 minutes of anaphase when the necessary conditions seem to exist. This time coincides with the beginning of phragmoplast formation.

In the fern, *Hymenophyllum*, a sublethal temperature applied at mid-anaphase increases the final distance between groups of sister chromosomes at late anaphase, i.e., the chromosomes move until they reach the cell walls. The effect occurs only in a narrow range of temperatures (42–47°C) and in a very limited time of late anaphase (Bajer and Molè-Bajer, 1953) coinciding with a rapid increase of birefringence in *Haemanthus* which denotes a change in MTs arrangement and/or number. The birefringence increases from the plate toward the pole (Inoué and Sato, 1967). At this stage, increasing numbers of MTs which were arranged in the form of arches begin to be superimposed or to overlap, and a number of straight segments also appear. It is only after this stage that interdigitation of MTs in the region of the cell plate occurs (i.e., mid or late stage of phragmoplast formation). Whether interdigitation occurs to some extent in earlier stages can be determined only after exact data on the number of MTs are obtained. The impression is that MTs break

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FIG. 6. Lateral movements during the beginning of phragmoplast formation in *Haemanthus katherinae* endosperm. The long arrow indicates the future position of the cell plate; dictyosomes are marked by short arrows. Due to lateral movements MTs bend into arches toward the outside of the phragmoplast. *Insets:* LM photomicrographs of the living cell *in vitro* and after embedding in plastic.

up in the equatorial region, and this is followed by a rapid increase in their number, leading to the observed increase in birefringence. The timing of the MTs' breakup, as far as can be judged by comparison of time-lapse film with fine structure, is precisely correlated with the time of exceptional movements by fragments toward the plate. Consequently, it may be speculated that MTs which break then grow rapidly toward the plate and push fragments with them. The movement of fragments to the plate occurs before the rapid increase of birefringence, which is undoubtedly correlated with an increase in the number of MTs and their probable growth away from the cell plate toward the poles. The latter conclusion is supported by the subsequent behavior of the fragments, which may stay at the cell plate for a short time and then tend to be eliminated toward the poles (Bajer and Molè-Bajer, 1963). The time of elimination corresponds with the stage when the rapid increase of birefringence occurs. The breakup of MTs and the interdigitation which follows occur close to the equator; it seems logical, therefore, that during the increase of birefringence MTs grow toward the poles, i.e., in the direction toward which fragments are eliminated. It is evident that the processes during late anaphase and early telophase are very complicated and not well understood.

According to most observors, the cell plate in its initial stages is formed from vesicles arising from the dictyosomes or Golgi bodies (GB); it is also possible that ER contributes to the cell plate. Hepler and Jackson (1968) suggested that ER is primarily responsible for cell plate formation in *Haemanthus* endosperm, but an independent examination of the same material indicated that a majority of the vesicles arise from the GBs (Bajer, 1968b). It is possible that the apparent disagreement between these two observations is due to differences in the methods of fixation. Although it has been argued that GBs are localized mostly at the polar ends and in regions around the phragmoplast and therefore cannot contribute to cell plate formation within the phragmoplast, these observations are not correct. *In vitro* studies of single GBs using Nomarski optics and confirmed by EM studies (Bajer and Molè-Bajer, unpublished observations) indicate that GBs are often found in the center of the interzonal region where they move and oscillate (Fig. 7). A change of position from parallel to perpendicular to the LM optical axis may take only 1–2 seconds. The problem of the mechanism responsible for the change of orientation and movements of GB vesicles will be discussed elsewhere (Bajer and Molè-Bajer, 1971). In any case, a few GBs can produce more than enough vesicles to form the complete cell plate. At some stage MTs are probably responsible for the transport of these vesicles to the plate; in the LM, clusters of vesicles appear

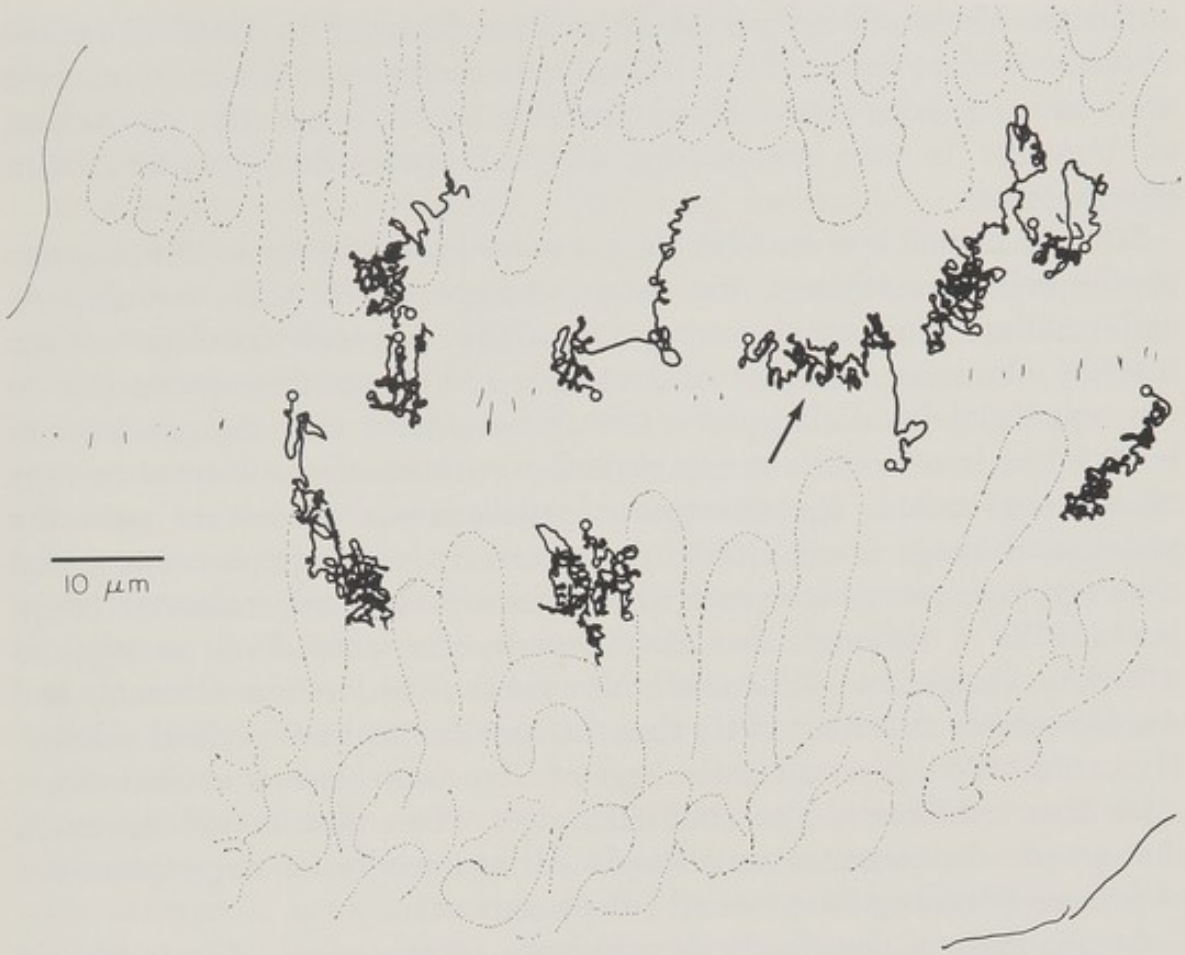


FIG. 7. The paths of dictyosomes (Golgi bodies) during late anaphase in *Haemanthus katherinae* endosperm. This drawing from time-lapse film covers a period of about 15 minutes during the contraction of chromosomes which marks the beginning of telophase. Each white circle is the beginning of an observed path of a dictyosome. Paths of several dictyosomes are superimposed in the film, but for clarity only one such case is shown (arrow), and the paths of only a few selected dictyosomes have been drawn.

as small droplets, which are seen sliding around MTs at this stage (Bajer, 1965). However, Hepler and Newcomb (1967) reported that they did not find any special relation between the distribution of MTs and the small vesicles. Some speculations about the important question of how MTs may transport vesicles during cell plate formation are given in Section XII.

B. Animal Cytokinesis

In animal cells the transition between anaphase and telophase has not been analyzed in as much detail as in plant cells. For example, it is not known whether fragments move toward the cell equator at the end of

anaphase. Movements of univalents will be discussed in detail elsewhere (Bajer and Molè-Bajer, 1971). Lateral movements by trailing chromosome arms have not been reported and probably do not occur. The mechanism of cleavage is also completely different from the formation of a phragmoplast.

In most animal species MTs do not contribute directly to the cleavage of the cell; nevertheless, the asters (composed of MTs, membranes, and vesicles) seem to play some role if they are well developed. They may be active in transport of materials (FMs) or their precursors to the equatorial band. Rapport (1969a) has found that the presence of both asters is necessary to establish the position of the furrow in eggs of the sea urchin, *Haemicentrotus pulcherrimus*. However, once the plane of cleavage is established the asters are no longer necessary, and cleavage then proceeds even if the asters are surgically removed (Rapport, 1969b). Although the latter experiments were done on eggs of a variety of species (*Echinarachmius parma*, *Hydractina echinata*, and *Aurelia aurita*) it is most likely that the conclusions have general validity. Hiramoto (1965) showed in the eggs of *Clypeaster japonicus* (first cleavage) that cytokinesis also continues even when, just before its onset, the spindle disintegrates or is displaced by injection of large quantities of sucrose (disintegration) or oil (displacement).

At anaphase a stem body or mid-body composed of MTs is formed from the remnants of continuous spindle MTs. The role of the stem body in chromosome distribution is not clear, but it very likely prevents the approach of chromosome groups toward each other at late anaphase and in this way its function is similar to that of the phragmoplast. It may also play an important role in the mechanism of the distribution of sex univalents (Bajer and Molè-Bajer, 1971). In microsurgical studies by Carlson (1952) and by Nicklas and Steahly (1967) it was possible to move a needle freely in the interzonal region, suggesting the absence of continuous fibers. However, if MTs in the interzonal region were broken or bent under experimental conditions, these results would be explained; this is not unlikely since in many microsurgical experiments the needle does not pierce the cell membrane.

At an early stage of stem body formation, time-lapse films show that a sideways movement of thin fibrils or birefringent streaks occurs. Somewhat later, the MTs exhibit a clear interdigitation in the central region of the stem body, similar to that in the phragmoplast. Since the transport properties of the stem body have been little studied (Byers and Abramson, 1968), it is not known whether elimination properties are of general occurrence. However, in time-lapse films of cells with large chromosomes, e.g., newt cells filmed by Zirkle (1957), it is clearly seen in some cells that small granules are transported toward the poles away from the center

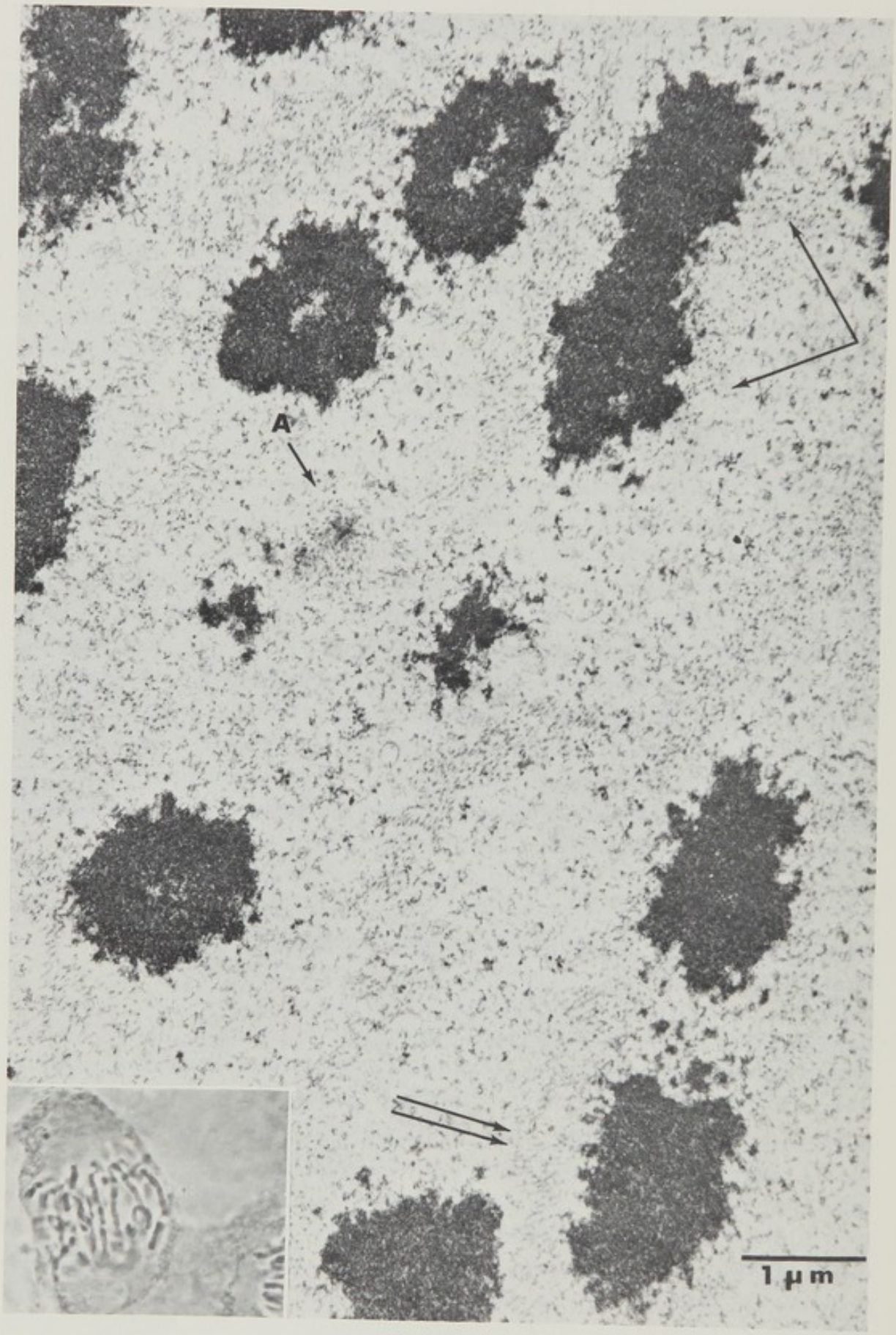
of the mid-body. This represents an additional similarity between the phragmoplast and stem body; it would not be surprising if electron microscopy revealed that this similarity is the result of activity by continuous fibers present in the interzonal region.

Animal eggs can be induced to cleave experimentally by centrifugation combined with hydrostatic pressure (Zimmerman and Marsland, 1956). Marsland *et al.* (1960) suggested that both nuclear and cytoplasmic factors are important for cleavage, and later Marsland and Landau (1954) concluded that cleavage results from the contraction of a jellified cortical cytoplasmic band around the cell equator. Very elegant experiments on the movement of cytoplasmic granules and carbon particles adhering to the surface during cleavage of eggs of the heart-urchin (*Clypeaster japonicus*) led Hiramoto (1958) to the same conclusion. More recently a number of electron microscopists have reported an accumulation of thin filaments about 40–60 Å in diameter in the cortical region (Szollosi, 1968, 1970; Schroeder, 1968; Tilney and Marsland, 1969; Arnold, 1969). This extends earlier observations that an electron dense region exists around the equators of cleaving eggs (Robbins and Gonatas, 1964; Mercer and Wolpert, 1957; Weinstein and Herbert, 1964). In a variety of unicellular species, thin filaments seem to be connected with contractile cytoplasm: e.g., in the pseudopodia of some amoebae (Wohlman and Allen, 1968), in contractile stocks of *Stentor*° (Randall and Jackson, 1958), and during cytoplasmic streaming in *Nitella* (Nagai and Rebhun, 1966). However, the actual mechanism of contraction and the role of the filaments in this process are not yet clear (see the article by McGee-Russell and Allen in this volume).

XI. Formation and Development of Spindle Fibers

The term "fiber" will be used for a bundle of MTs located close together and separate from other similar bundles. Individual spindle fibers of this sort can be seen with the LM, and three types have commonly been distinguished: (1) chromosomal fibers; (2) continuous fibers; and (3) interzonal fibers (Schrader, 1953). The term kinetochore fiber has been suggested for bundles of MTs connected to the kinetochore (Bajer, 1968c). Evidence also exists that interzonal fibers are remnants of continuous fibers in *Haemanthus* endosperm; these probably originate in most, if not all, higher plants from MTs found outside the prophase nucleus (i.e., in the CZ), which invade the nuclear region

° *Added in proof:* Huang (*J. Cell Biol.* **47**: 92a, 1970) showed that in *Stentor* a sliding system of MTs is involved during contraction.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after the BNE. It is not known where they end. The commonly accepted view is that some actually run from one pole to the opposite, while others end (or begin) somewhere between. To some extent the term "fiber" in relation to continuous fibers is misleading; aggregates of MTs forming continuous fibers, when seen in spindle cross sections, constitute sheets or rather irregular bands (Fig. 8). These fibers often fuse laterally, and consequently may form a continuous branched structure (in cross section). Because of this property, only some continuous fibers can be seen with the LM, and with the polarizing microscope they are seen as streaks of birefringent material. In prometaphase-metaphase the behavior of continuous fibers is better understood than that of kinetochore fibers, whereas the reverse is true in anaphase. The origin of kinetochore fibers seems to be more complex. In plants one sees that MTs are connected to the kinetochores a few minutes after the BNE; because no MTs are present inside the prophase nucleus, kinetochore fibers must develop rapidly after the BNE.

Detailed studies of *Haemanthus* endosperm, including both normal and chloral hydrate-treated cells show that, in the latter, kinetochore fibers are formed at the kinetochore and grow away from it (Bajer and Molé-Bajer, 1969; Molé-Bajer, 1967, 1969). In untreated normal cells, on the other hand, some MTs, from a CZ destined later to form continuous fibers, seem to attach directly to some kinetochores during BNE (Figs. 9 and 10). Kinetochores to which MTs do not attach during the start of prometaphase then form MTs very rapidly. It seems, therefore, that both kinetochores and the CZ MTs play a role in the formation of kinetochore fibers. The attractive speculation that all MTs forming kinetochore fibers always grow away from kinetochores along continuous fibers has not been fully supported by EM studies (Bajer and Molè-Bajer, 1969); however, this may be so if we assume that kinetochore MTs formation is exceptionally rapid. The number of MTs per kinetochore in the first stage is 10–20, but this increases rapidly to 70–150 in metaphase. It is logical to assume that the number increases because of the activity of the kinetochores, and this again indicates that kinetochore fibers have a dual origin. Inoué and Sato (1967) also suggested that continuous fibers may transform into kinetochore fibers, and vice versa.

FIG. 8. Cross section of anaphase half-spindle in *Haemanthus katherinae* endosperm. The section is approximately at the kinetochore level, and the arrow (A) points to sectioned kinetochore fibers just above a kinetochore. Angled arrows point to a cross section of a chromosome below the kinetochore. Continuous fibers form two sheets on both sides of the chromosome. MTs in some continuous fibers (two parallel arrows) are cut obliquely, indicating that the MTs are arranged at a different angle. *Inset:* LM photomicrograph of the same cell embedded in plastic.

In animal cells, few data are available concerning the fine structural processes following the BNE, and it is not known whether the continuous MTs attach to the kinetochores. Observations by Brinkley and Stubble-

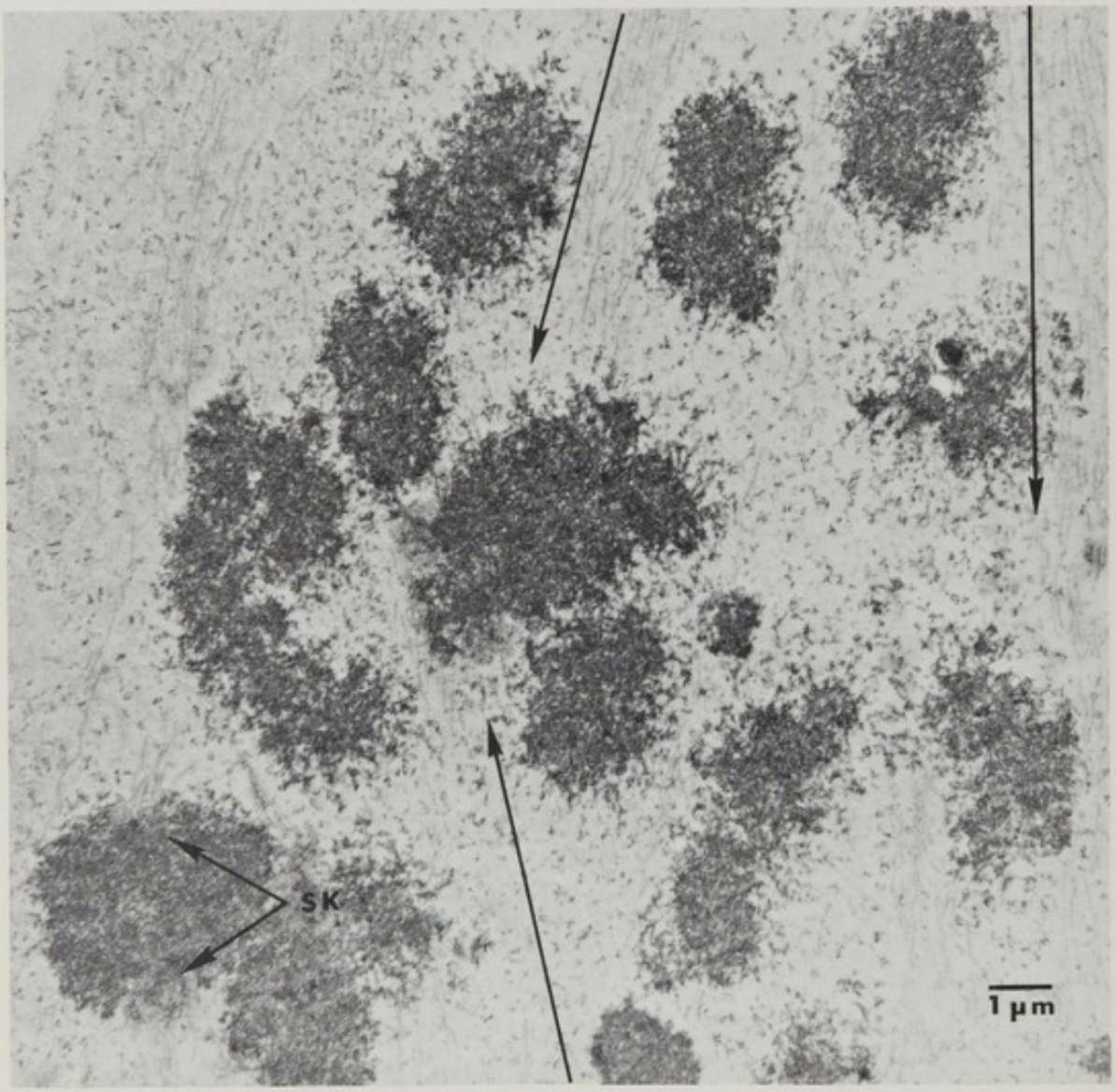

FIG. 9. Early prometaphase in *Haemanthus katherinae* endosperm. MTs from the CZ penetrate inside the nucleus (long arrows indicate the directions of penetration). Some sister kinetochores show the start of MTs arranged parallel to the direction of penetration from both poles (arrow SK). Very few MTs are connected with other kinetochores (Fig. 9, center, and Fig. 10). Those MTs which are connected seem to be continuous fibers. LM photomicrograph of this cell is given in Fig. 3.

field (1966) and Brinkley *et al.* (1967a) on the fine structure of the kinetochore in the Chinese hamster cells indicate that kinetochore fibers originate at the kinetochore. Additional evidence that kinetochore fibers form at the kinetochore and grow toward the pole comes from the observations of Forer (1965, 1966) using a UV microbeam. Because the

zone of reduced birefringence produced by irradiation moves away from the kinetochore toward the pole, the process can be interpreted as growth of MTs away from the kinetochores. Fine structure studies of a region irradiated by UV microbeam give some support to such an

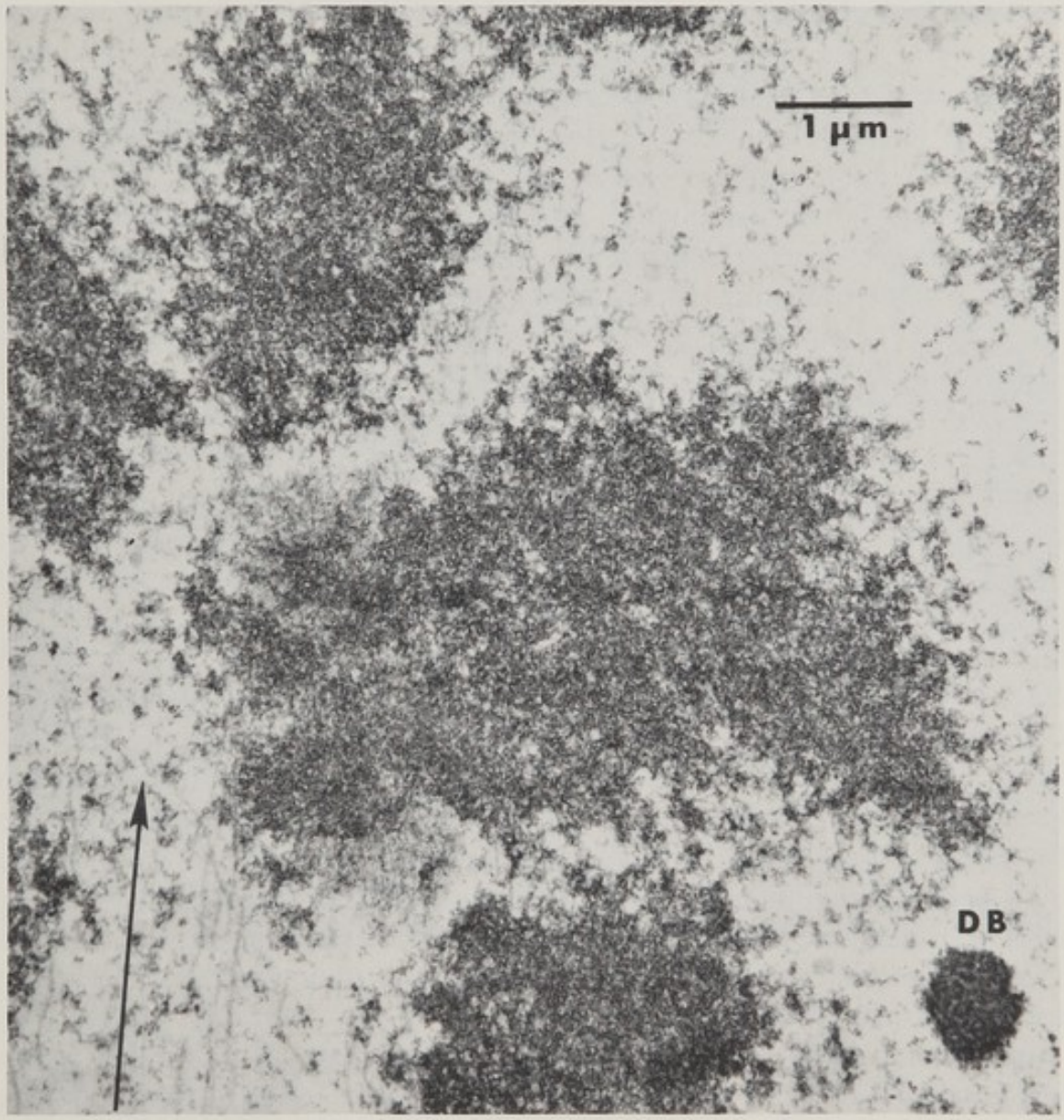

FIG. 10. Part of Fig. 9 at higher magnification. Dense bodies (DB) are formed in the nucleolus; these bodies persist throughout mitosis.

interpretation (Bajer and Molè-Bajer, 1971); if a proper dose of UV irradiation is applied to *Haemanthus* endosperm and the cell is fixed at an appropriate time after irradiation, MTs in the irradiated region are clearly damaged and broken into short segments which are irregularly arranged. MTs are then gradually reconstructed in the irradiated

spot. Often the only lasting effect is a reduction in the overall number of MTs.

The arrangement of MTs within kinetochore fibers changes during mitosis and is strongly influenced by mechanical conditions within a cell. In flattened *Haemanthus* endosperm cells, where the chromosomes are arranged almost in a single layer, kinetochore MTs forming a single kinetochore fiber diverge slightly toward the pole during prometaphase and metaphase (Fig. 11). As a rule this divergence increases in cells flattened to the same extent during anaphase (Fig. 12). Such changes in divergence exist, but are not as clear in half-flattened or unflattened cells; in the latter, the change of shape in single kinetochore fibers is usually slight. Very divergent kinetochore fibers can occasionally be seen at metaphase, but it is evident that the change in divergence is dependent on mechanical conditions in the spindle. Occasionally a change in divergence can be seen with the Nomarski system and with polarized light in living cells during anaphase; strongly birefringent fibers increase in diameter (Forer, 1965), and their birefringence per unit area decreases because the number of MTs found in a given area drops. However, the problem is complicated by probable changes in the number of MTs; another complication is a frequent tendency of kinetochore fibers (as whole units) to converge toward the polar region during metaphase and to increase this convergence during anaphase in all types of *Haemanthus* cells. Although no precise counts have been made in *Haemanthus*, it appears that the number of MTs connected with the kinetochores diminishes during anaphase. Kinetochore fibers decrease in length but short MTs (2–5 μ) are still attached to each kinetochore when the chromosomes stop their poleward anaphase movement. A few MTs may persist in *Haemanthus* for some time and be incorporated in the telophase nucleus, but MTs have not been observed in prophase nuclei. The various factors responsible for the shapes of kinetochore fibers are not yet entirely clear.

In animal cells and in several lower organisms during metaphase-anaphase, MTs in the kinetochore fibers are arranged more or less parallel, and all fibers (understood to be bundles of MTs) converge toward the pole—often precisely toward the centriole both at metaphase and in late anaphase. Irrespective of changes in divergence-convergence, the regular arrangement of MTs gradually disappears and MTs in late anaphase are irregularly arranged in the polar regions. In the crane fly, Behnke and Forer (1966) observed MTs inside prophase and telophase nuclei. It might be speculated, therefore, that in some cells MTs persist from one division to the next; it would be of special interest to follow the contact between MTs and kinetochores in such cells. The

idea that spindle fibers persist was suggested by Lettré and Lettré (1958), but so far no experimental support has been obtained.

During the entire process when chromosomes move, the kinetochore MTs intermingle with continuous MTs; such continuous fibers have been found in all dividing cells studied so far. Intermingling is especially clear in the polar regions where, unfortunately, it is not usually possible

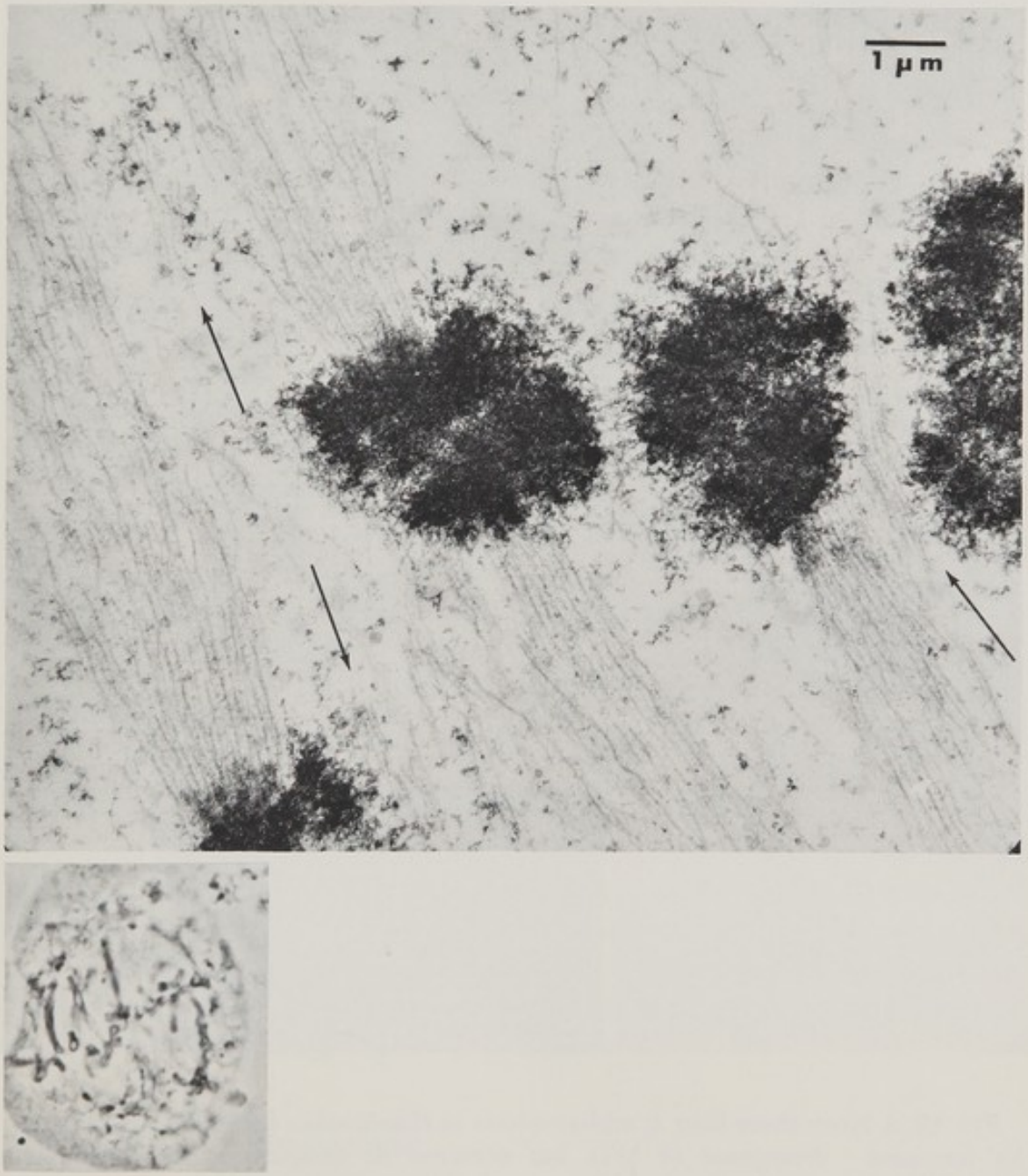

FIG. 11. Metaphase in *Haemanthus katherinae* endosperm. In this half-flattened cell, the developing kinetochore fibers diverge slightly toward the pole. Continuous and kinetochore fibers are arranged parallel to each other (arrows; cf. Fig. 13). Denser and lighter components of kinetochores are clearly visible. *Inset:* LM photomicrograph of the same cell embedded in plastic.

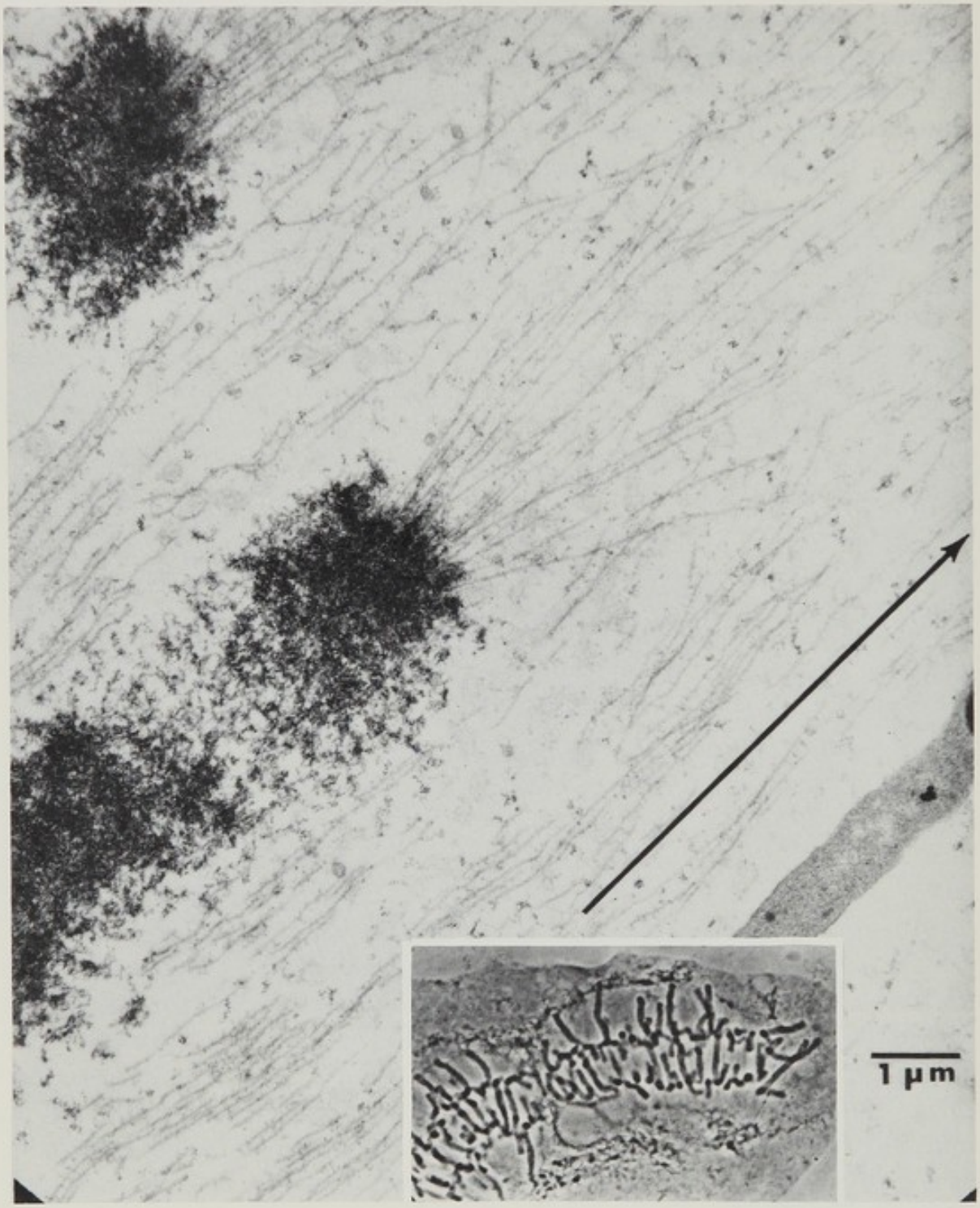

FIG. 12. A kinetochore fiber at mid-anaphase in *Haemanthus katherinae* endosperm. An increased divergence of MTs has occurred in comparison with metaphase (Fig. 11). Several continuous MTs are seen (arrows); however, numerous MTs were also found on the surface of this cell (not reproduced here). *Inset:* LM photomicrograph of the same cell embedded in plastic. Part of this cell not visible in the insert was irradiated with a UV microslit, and the cell was fixed 1 minute after irradiation; no detectable changes in MT arrangements were found in non-irradiated region (Bajer and Molè-Bajer, 1971).

to distinguish continuous MTs from kinetochore MTs. However, Allenspach and Roth (1967) found only a few continuous MTs in cells which do not elongate; they are also very scarce in cells with strongly developed membranes around the spindle, like honey bee spermatocytes (Hoage and Kessel, 1968). In general, continuous MTs form a mantle around the spindle, but such a mantle is not present or is very weakly developed when a system of membranes develops around the spindle. It might be suggested in such cases that the membranes are a substitute for the strong development of continuous MTs.

Numerous observations indicate that some MTs pass through the chromosome arms, usually in the peripheral regions. In *Haemanthus,* however, detailed measurements of chromosome shrinkage during dehydration suggest that most such MTs may in fact lie on the surface of the chromosome and are not embedded inside the chromosome in living cells. Nevertheless, some MTs are found passing *deep inside* the chromosome, as first reported by Robbins and Gonatas (1964) and confirmed by others in several other species. The role of such MTs penetrating the chromosome body is not clear. It might be speculated that some of these MTs could be responsible for neocentric activity, but others do not seem to play any particular role.

XII. Spindle Fine Structure and the Mechanism of Chromosome Movements

The main questions which should be explained by any general hypothesis of the mitotic mechanism are: (1) How is the "pull" on kinetochores effected and how does it influence chromosome behavior? (2) What mechanism eliminates unattached bodies out of the spindle (i.e., behavior of acentric bodies and small granules)? (3) What causes elongation of the spindle? Only some aspects of these problems will be presented here. A more extensive discussion is given elsewhere (Bajer and Molè-Bajer, 1971). Cytokinesis is regarded as a separate problem and will not be discussed again here (see Section X).

A. The Pulling Mechanism

How the pull occurs is one of the most essential questions. In the authors' opinion, it is most useful at present to regard "pull" and "elimination" as two aspects of the same mechanism. During the majority of movements kinetochore fibers pull kinetochores; however, because

MTs are not attached to the poles in either plants or animals, the question arises: "How can any pull take place at all?" Various explanations can be suggested from LM and EM observations; because there is evidently a precise correlation between the number of MTs (EM) and the degree of birefringence (LM; Sato, personal communication), MTs can be seen with the LM and followed in living cells (Bajer and Allen, 1966; Bajer and Jensen, 1969). The suggestion by Forer (1966) that the birefringent components of kinetochore fibers do not transmit the pulling force is certainly not correct; microsurgical experiments by Carlson (1952), Nicklas and Steahly (1967), and Nicklas (1967) show this especially clearly.

1. *Side Arms of MTs*

Because MTs are kept in tension, but seem not to be fixed at the polar regions, the most tenable alternative is that they are fixed to the spindle all along their length or along a substantial part. Forer (1965, 1966) suggested that between one half and one third of each fiber is enough to move the chromosomes. From data presently available, the only structural element deserving serious consideration as attaching MTs are side arms of MTs. Although side arms are not usually seen distinctly, when the MTs are arranged in parallel, as during the formation of the phragmoplast (Fig. 13), side arms are often rather regularly spaced by means of difficult-to-define lateral connections. Such connections between MTs have long been known in cilia and flagella (see review by Gibbons, 1968). Furthermore, an increasing number of observations indicate that different types of MTs exist; Behnke and Forer (1967) mention four (see also Stephens, 1970). Strikingly regular interconnections between MTs are found occasionally in other organelles, especially if they have a highly specialized function. For example, six connections have been seen in the ciliate *Nassula*, resulting in a precise spatial arrangement of MTs (Tucker, 1968; see also Tilney and Marsland, 1969; Manton, 1964). Connections in the form of short arches or bridges are often found in nondividing cells, such as the leaf glands of *Phaseolus vulgaris* (O'Brien, 1967), as well as in dividing cells (Wilson, 1968, 1969; McIntosh, 1968; Hepler and Jackson, 1968; Bajer and Molè-Bajer, 1969). It has been speculated that such connections may be very regularly arranged around the MTs and form a helix with a bridge every 30° (McIntosh, 1968).

Side arms are key elements in the sliding MT hypothesis of McIntosh *et al.* (1969), who suggested that the presence of arms determines the orientation and polarity of MTs. They also suggested that

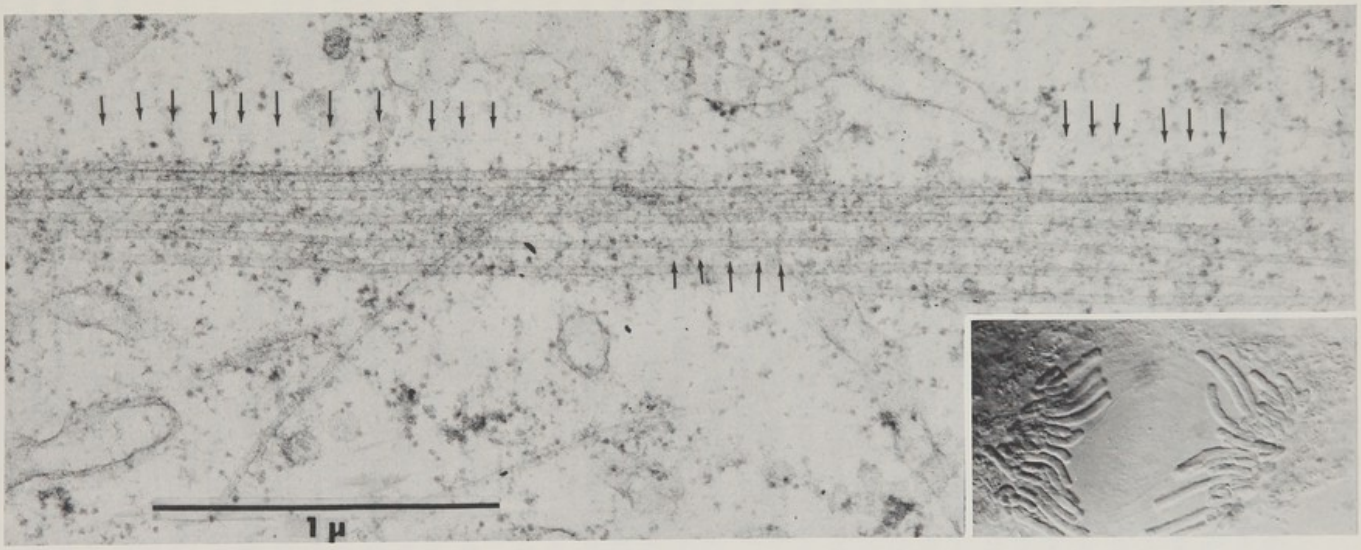

FIG. 13. Side arms of MTs during early stages of phragmoplast development at a time in early telophase when the chromosomes have just begun to contract. MTs arranged parallel to each other are seen to be connected by diffuse structures (side arms) which are rather uniformly spaced (arrows). These structures are seen on the outer MTs also. *Inset:* LM micrograph of the same cell embedded in plastic (Nomarski system). The cell showed strong lateral movements and the fibrils seen with the LM were bent sideways.

if the arms in neighboring MTs point in opposite directions, the MTs slide past one another. According to this hypothesis, there are two main systems of MTs: continuous ones and kinetochore MTs, which move past one another. However, observations on transport activity within the spindle do not agree with this intepretation. Also exact counts of numbers of MTs (Brinkley, unpublished) are not in accord with this model. To account for the discrepancy and to explain elongation of the spindle, the authors postulated that continuous MTs also slide past one another. They suggested that one end of an MT is embedded in a dense amorphous structure around the centriole. During anaphase these MTs slide toward the center of the stem body and form the equatorial region where interdigitation occurs. However, other observations indicate that the dense regions in the stem body may form in a different way. Another problem arises from data in the literature which indicate that bridges between MTs, when they occur, are found in structures which do not permit any movement of MTs; rather, they tend to anchor or bind MTs more firmly together. Finally, the transport properties within the continuous fibers seem to be the same as in kinetochore fibers (Allen *et al.*, 1969). Although several investigators have suggested that transport of MTs occurs during mitosis (Jenkins, 1967; Bajer, 1968a; Wilson, 1969), not enough data are available yet to establish the detailed hypothesis proposed by McIntosh *et al.* (1969). The nature and role of arms, and even their occurrence in relation to fixation, remain to be clarified.

2. *The Role of MFs*

Another, more remote possibility is that MFs play some role in keeping MTs in tension. MFs are important during cleavage (Schroeder, 1968; Arnold, 1969; Tilney and Marsland, 1969). They have also been found occasionally in the spindle of *Haemanthus* at different stages (Bajer and Molè-Bajer, 1969). However, they have not been reported generally, and it is difficult to judge at present whether they were not present or simply not found. In any case, they do not form easily visible bundles in the spindle in the majority of objects studied so far.

3. *Activity of MTs. Conclusions*

At all stages the MTs of the spindle show a decreasing orderliness from the kinetochores to the poles, a feature which is especially clear in the polarizing microscope. If this gradient of orderliness is associated with biochemical or biophysical differences, this alone may be sufficient to keep MTs in tension, e.g., diffusion forces would probably be able

to anchor MTs along their entire lengths. Motive forces based on membrane charges (electroosmosis; Ambrose and Forrester, 1968) also deserve more experimental study. Evidence of a gradient in spindle tension reported by Stich (1954) and in studies by Nicklas and Koch (1969) may be interpreted in this way.

It can be concluded that a variety of factors may be responsible for anchoring MTs and thus permitting a pulling force; it is not clear at present whether in actuality a single factor or several of them do anchor and keep MTs in tension. Lack of clear understanding about this question makes answers to another one, i.e., where the pull is located, especially speculative. For example, events resulting in pull may be located close to the pole, at the kinetochore, or along the entire length of the fiber. Östergren *et al.* (1960) suggested the latter alternative, and this view was shared by Subirana (1968); birefringence studies, especially during anaphase when kinetochore fibers decrease in birefringence, also led Inoué and Sato (1967) to a similar conclusion. At a molecular level, the simplest explanation would be that exchange of MT subunits along the whole length of a kinetochore fiber occurs due to intussusception (i.e., the insertion of molecules along the entire length of an MT). However, the majority of fine structure studies on MTs do not support this speculation, leading to a clear disagreement between conclusions based on different evidence. It is well documented and generally accepted that the spindle exhibits primarily a form birefringence. Consequently, changes of birefringence may be caused by changes of MTs orientation, decreases of MTs number, or a combination of both these factors. Data on *Haemanthus* and the Chinese hamster (Brinkley *et al.*, 1969) suggest that the latter is more nearly correct.

The fanning out of MTs in anaphase cannot produce any measurable decreases in length of kinetochore fibers. Therefore, three possibilities exist: (1) MTs decrease in length at the kinetochore, (2) MTs decrease in length at the pole, and (3) MTs decrease in length uniformly along the whole axis.

On the basis of freeze-etching studies, Moor (1966, 1967) found MTs of three basic diameters in yeast cells. He suggested that the pitch under which MT subunits are arranged and the number of subunits per turn may vary as MTs shorten along their entire lengths. In *Psilotum*, MTs of different thicknesses (180 and 280 Å) were found in prophase but not at later stages (Allen and Bowen, 1966). Some differences in the thickness of MTs during prometaphase, metaphase, and anaphase were found in animal cells by Allenspach and Roth (1967). However, not enough data have been accumulated to state whether this occurs in most other species, and most investigators do not believe that MTs

shorten uniformly along their whole lengths. An elegant experiment by Moses *et al.* (1968) has proven that one end of a growing MT in a flagellum has the appearance of a dense amorphous body; similar structures have been found in the *Haemanthus* phragmoplast (Bajer, 1968b). Furthermore, the region of a kinetochore where the MTs are embedded is very similar to the dense amorphous bodies mentioned above at least in *Haemanthus*. It may be speculated, on the one hand, that MTs increase in length at the kinetochore, at the pole, or at both ends; on the other hand, if the dense bodies are characteristic for growing ends of MTs, then the MTs may grow at the kinetochore and be disorganized at the pole. The latter possibility is highly speculative, but it is especially attractive because it might explain the elimination properties of the spindle. Thus, if we assume that kinetochore MTs grow continuously at the kinetochore toward the pole during prometaphase-metaphase, then the material to make MTs must be added at the kinetochore and disposed of at the pole; in this way, material forming MTs and some material adjacent to MTs might be eliminated toward the pole. Since MTs occupy about 5–10% of the volume of the spindle, the spindle would decrease slightly in size during metaphase, a phenomenon which is actually observed. In this model, kinetochore MTs would move toward the pole during prometaphase-metaphase, taking with them small particles, acentric bodies, and straightened chromosome arms. An important aspect of elimination, i.e., constant speed of eliminated bodies independent of their size, would be fulfilled. Finally, the poleward movement of a zone of reduced birefringence can be regarded as comparable to the elimination of acentric bodies and explained in the same way (Forer, 1965, 1966).

If it is really true that kinetochore MTs grow poleward during prometaphase-metaphase, then it follows that this growth must stop at the beginning of anaphase; the timing of elimination would be in accord with this supposition, as well as the fact that small particles embedded in the kinetochore fibers move in front of the kinetochore during anaphase so that the distance between them and the kinetochore remains constant (Bajer and Molè-Bajer, 1963; Bajer, 1968a). During anaphase, kinetochore MTs evidently decrease in length at the poles and entire kinetochore fibers are transported poleward. The movement of entire kinetochore fibers can be seen clearly in time-lapse films (Bajer, 1968c), especially if the diffraction pattern of the spindle fibers is analyzed (Allen *et al.*, 1969). Such an interpretation was suggested earlier by observations of kinetochore fiber birefringence (Bajer, 1961), and it implies that each kinetochore fiber is transported (eliminated) rather passively poleward during anaphase. The motive force may be an inter-

action between kinetochore fibers and continuous fibers (Bajer, 1968a,c), implying that there are two systems of MTs with different properties. Although one system with special properties would be sufficient (Subirana, 1968), evidence for the existence of two systems (continuous and kinetochore MTs) is provided by the action of colcemid, which disorganizes continuous MTs first and then later affects kinetochore MTs (Molè-Bajer, unpublished observations). On the other hand, as the movements within continuous and kinetochore fibers seem to be the same (Allen *et al.*, 1969), this problem is not yet clear and needs further studies.

Another possible mechanism for spindle elongation, would be the sliding of continuous fibers past one another (McIntosh *et al.*, 1969). However, precise counts of MTs in anaphase do not support this mechanism unless additional assumptions are made (Brinkley *et al.*, 1969; McIntosh, 1968). In addition, data about the origin and growth of the phragmoplast make such suggestions highly improbable in plants (see Sinnott and Bloch, 1941; Bajer, 1965, 1968c). It would be expected that different MTs within continuous fibers must be moving in opposite directions, but it is very unlikely that such movements would not have been detected during precise analysis of continuous fiber activity (Allen *et al.*, 1969). An alternative possibility would be that continuous MTs increase in length at the equatorial region during anaphase. Since chromosomes are still able to move in chloral hydrate-treated cells, where no clear continuous fibers exist, other structures may act to support or anchor the kinetochore fibers (e.g., kinetochore fibers of neighboring chromosomes, membranes). Still another possibility is that the MTs themselves may create conditions for their own transport; this was strongly argued for kinetochore fibers by Östergren *et al.* (1960; see also Subirana, 1968). It is evident that various explanations can be given at present, all of which face some difficulties and none of which can be considered as proven.

B. Elongation

Elongation of the spindle in anaphase may involve an increase in the length of the continuous MTs, or their sliding against each other (McIntosh *et al.*, 1969). The former was indicated by Roth *et al.* (1966) and by Allenspach and Roth (1967), who found that continuous fibers are scarce in cells which elongate slightly and more abundant in those which elongate considerably. Because continuous fibers are rare in some cells, it may not be surprising that their presence was not evident

in microsurgical studies (Carlson, 1952; Nicklas and Steahly, 1967). Elongation of continuous MTs may even push the spindle poles apart (Bajer, 1968a), but it is not clear how this elongation occurs at the fine structure level. If the end of a growing MT is a dense amorphous structure, no such structures have been found during anaphase at the poles (except for satellites near the centrioles). On the other hand, such dense structures begin to be found during anaphase in the central or interzone region in *Haemanthus* (Bajer, 1968c), and they have been described at a later stage in animal cells. Allenspach and Roth (1967) suggested that this might be the region where MTs increase in length by intussusception, and such an interpretation has been supported by Robbins and Jantzsch (1969). The fine structural mechanism is not clear, however, and may be either intussusception or MT breakage followed by poleward growth.

XIII. A General Hypothesis

Although the discussion of chromosome pull and transport given in previous sections is highly speculative, the various principles developed are sufficient to explain different disturbances and abnormalities [including the behavior of acentric fragments, univalents, and monopolar divisions such as *Sciara* (Metz, 1933)]. More detailed discussion especially of asters in chromosome movements, is presented elsewhere (Bajer and Molè-Bajer, 1971). The main principles may be summarized as follows:

1. Centrioles and asters do not play an essential role in standard chromosome movements and spindle formation, although they may help to organize the spindle and may play some role in cytokinesis.

2. The important role of the asters is to prevent accidental loss of centrioles and possibly to determine the plane of cleavage.

3. Kinetochore MTs grow continuously at the kinetochores during prometaphase and metaphase, and stop growing during anaphase. During poleward growth they carry with them small particles, acentric bodies, etc; consequently, the elimination properties of the spindle depend both on the activity of MTs and on their growth.

4. The metaphase plate is formed as a result of "pulling" the kinetochores toward opposite poles according to principles outlined previously (Östergren *et al.*, 1960). Several secondary factors, such as mechanical conditions in the cell, properties of chromosomes, activity of kinetochores, also play some role in this process.

5. Kinetochore fibers are transported (eliminated) toward the poles

during anaphase. They are progressively disorganized at the polar regions.

6. Elongation of the spindle occurs due to an increase in length of continuous MTs (at the poles, at the equator, or both).

7. Cytokinesis (formation of a phragmoplast or animal cleavage) is a separate process, but may be triggered by chromosome movements.

The above points are regarded, not as a theory, but as a useful working hypothesis. Nevertheless, several experiments and facts seem to support these suggestions, and they have the advantage that they can be checked further by appropriate experiments. In the authors' opinion, there are not yet sufficient data to propose a molecular basis for chromosome movement and transport phenomena.

XIV. Problems for the Future

A vast amount of information on various aspects of cell division has been accumulating. We have discussed data related especially to fine structure and have attempted to draw attention to a few selected morphological aspects of chromosome movements. The importance of the actual function of the mitotic spindle ("pull" and "elimination") has been emphasized; an understanding of this problem may explain the variety in chromosome behavior. Several essential problems were omitted intentionally, e.g., energetics and cytochemistry of the mitotic apparatus, because we believe that the structural aspects must be well known before any significant understanding of energetics is possible. Some problems, e.g., chromosome orientation, behavior of sex chromosomes, the role of asters etc., were also discussed very shortly.

It has become apparent in the last few years that MTs are essential for distribution of the chromosomes, and MFs for cleavage. In the authors' opinion, more detailed information is needed about certain fine structural and physiological aspects of MTs. Observations on the behavior of MTs in living cells are likely to supply important clues. In particular, the following essential questions need to be answered: How long are continuous and kinetochore MTs? Where do they end in the spindle? Does the number of MTs change during mitosis and if it does, how and where? What material surrounds the MTs—side arms or some other structures? If it represents side arms, what is their function?

Undoubtedly, studies of some deviant types of cell division will permit us to understand these processes and also to eliminate errors which

have persisted in the literature for a long time. It is rather unfortunate that various fine structural reports are lacking in detail, and the authors have not included information which, in some cases, has been obtained by experiments but not described. We hope that various remarks in this article will draw attention to problems deserving attention in the future.

In conclusion, we wish to emphasize that Schrader's (1953) approach to this problem is still valid, and if followed, would avoid confusion and erroneous explanations based on inadequately founded facts. He pointed out: "The attitude of the past that the answer will some day be revealed to us by a stroke of genius or by luck is almost certainly illusory. The solution will be approached much more surely if we make up our minds that we are confronted by some painstaking work that has to be guided by intelligent planning and a thorough knowledge of the results so far obtained."

Acknowledgments

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REFERENCES

Allen, R. D., and Bowen, C. C. (1966). *Caryologia* **19**: 299

Allen, R. D., Bajer, A., and LaFountain, J. (1969). *J. Cell. Biol.* **43**: 4.

Allenspach, A. A., and Roth, L. E. (1967). *J. Cell Biol.* **33**: 179.

Ambrose, E. J., and Forrester, J. A. (1968). "Aspects of Cell Motility." *Symp. Soc. Exp. Biol.* **22**: 237.

Arnold, J. M. (1969). *J. Cell Biol.* **41**: 894.

Bajer, A. (1961). *Chromosoma* **12**: 64.

Bajer, A. (1965). *Exp. Cell Res.* **37**: 376.

Bajer, A. (1966). *In* "Probleme der biologischen Reduplikation" (P. Sitte, ed.), p. 90. Springer-Verlag, New York.

Bajer, A. (1968a). Aspects of Cell Motility. *Symp. Soc. Exp. Biol.* **22**: 286.

Bajer, A. (1968b). *Chromosoma* **24**: 383.

Bajer, A. (1968c). *Chromosoma* **25**: 249.

Bajer, A., and Allen, R. D. (1966). *Science* **151**: 572.

Bajer, A., and Jensen, C. (1969). *J. Microsc. (Paris)* **8**: 124.

Bajer, A., and Molè-Bajer, J. (1953). *Acta Soc. Bot. Pol.* **22**: 477.
Bajer, A., and Molè-Bajer, J. (1963). *In* "Cinemicrography in Cell Biology" (G. G. Rose, ed.), p. 357. Academic Press, New York.
Bajer, A., and Molè-Bajer, J. (1969). *Chromosoma* **27**: 448.
Bajer, A., and Molè-Bajer, J. (1971). *Int. Rev. Cytol.* (In Press).
Bajer, A., and Östergren, G. (1961). *Hereditas* **47**: 563.
Bajer, A., and Östergren, G. (1963). *Hereditas* **50**: 179.
Behnke, O. (1967). *J. Cell Biol.* **34**: 697.
Behnke, O., and Forer, A. (1966). *C. R. Trav. Lab. Carlsberg* **35**: 437.
Behnke, O., and Forer, A. (1967). *J. Cell Sci.* **2**: 169.
Bělař, K., and Huth, W. (1933). *Z. Zellforsch. Mikrosk. Anat.* **17**: 51.
Beyer, H. (1966). *Jenaer Jahrb.* p. 173.
Bibring, T., and Baxandall, J. (1969). *J. Cell Biol.* **41**: 577.
Bleier, H. (1930). *Cellule* **40**: 85.
Bloom, W. (1960). *J. Biophys. Biochem. Cytol.* **7**: 191.
Braselton, J. P., and Bowen, C. C. (1969). *J. Cell Biol.* **43**: 169a.
Brinkley, B. R., and Stubblefield, E. (1966). *Chromosoma* **19**: 28.
Brinkley, B. R., Stubblefield, E., and Hsu, T. C. (1967a). *J. Ultrastruct. Res.* **19**: 1.
Brinkley, B. R., Murphy, P., and Richardson, L. C. (1967b). *J. Cell Biol.* **35**: 279.
Brinkley, B. R., Cartwright, J., and Murphy, P. (1969). *J. Cell Biol.* **43**: 169a.
Buck, R. C. (1967). *J. Ultrastruct. Res.* **18**: 489.
Burgess, J., and Northcote, D. H. (1967). *Planta* **75**: 319.
Burgess, J., and Northcote, D. H. (1968). *Planta* **80**: 1.
Butterfass, T. (1969). *Planta* **84**: 230.
Buvat, R. (1963). *Int. Rev. Cytol.* **14**: 41.
Byers, B., and Abramson, D. H. (1968). *Protoplasma* **66**: 413.
Carlson, J. G. (1938). *Proc. Nat. Acad. Sci. U. S.* **24**: 500.
Carlson, J. G. (1952). *Chromosoma* **5**: 199.
Comings, D. E. (1968). *Amer. J. Hum. Genet.* **20**: 440.
Cornman, I. (1944). *Am. Naturalist* **78**: 410.
Costello, D. P. (1961). *Biol. Bull.* **120**: 285.
Cronshaw, J., and Esau, K. (1968). *Protoplasma* **65**: 1.
Davies, H. G., and Tooze, J. (1966). *J. Cell Sci.* **1**: 331.
de Harven, E., and Bernhard, W. (1956). *Z. Zellforsch. Mikrosk. Anat.* **45**: 378.
Dietrich, J. (1966). *C. R. Acad. Sci., Ser. D* **262**: 1699.
Dietz, R. (1956). *Chromosoma* **8**: 183.
Dietz, R. (1959). *Z. Naturforsch. B* **14**: 749.
Dietz, R. (1966). *Chromosomes Today, Proc. 1st Oxford Chromosome Conf., 1964* **1**: 161.
Dietz, R. (1969). *Naturwissenschaften* **56**: 237.
Dirksen, E., and Crocker, T. T. (1966). *J. Microsc. (Paris)* **5**: 629.
Esau, K., and Gill, R. H. (1969a). *Can. J. Bot.* **47**: 581.
Esau, K., and Gill, R. H. (1969b). *Virology* **38**: 464
Feldherr, C. M. (1965). *J. Cell. Biol.* **27**: 28a.
Forer, A. (1965). *J. Cell Biol.* **25**: 95.
Forer, A. (1966). *Chromosoma* **19**: 44.
Forer, A., and Goldman, R. D. (1969). *Nature (London)* **222**: 689.
Franke, W. (1970). *Z. Zellforsch. Mikrosk. Anat.* **105**: 405.

Gibbons, I. R. (1968). *Annu. Rev. Biochem.* **37**: 521.
Hancock, R., and Ryser, H. (1967). *Nature (London)* **213**: 701.
Harris, P. (1962a). *J. Cell Biol.* **14**: 475.
Harris, P. (1962b). *Electron Microsc., Proc. 5th Int. Congr., Philadelphia.* Academic Press, New York.
Harris, P., and Mazia, D. (1962). *In* "The Interpretation of Ultrastructure," Symp. Int. Soc. Cell Biol., Vol. 1, p. 279. Academic Press, New York.
Heist, H. E., and Mulvanay, B. D. (1968). *J. Ultrastruct. Res.* **24**: 86.
Heslop-Harrison, J. (1968). *J. Cell Sci.* **3**: 457.
Hepler, P., and Jackson, W. T. (1968). *J. Cell Biol.* **38**: 437.
Hepler, P., and Newcomb, E. (1967). *J. Ultrastruct. Res.* **19**: 498.
Hiramoto, Y. (1958). *J. Exp. Biol.* **35**: 407.
Hiramoto, Y. (1965). *J. Cell Biol.* **25**: 161.
Hoage, T. R., and Kessel, R. G. (1968). *J. Ultrastruct. Res.* **24**: 6.
Hoskins, G. C. (1969). *Caryologia* **22**: 229.
Hsu, T. C., Brinkley, B. R., and Arrighi, F. E. (1967). *Chromosoma* **23**: 137.
Inoué, Shinya, and Bajer, A. (1961). *Chromosoma* **12**: 48.
Inoué, Shinya, and Sato, H. (1967). *J. Gen. Physiol.* **50**: 259.
Jenkins, R. A. (1967). *J. Cell Biol.* **34**: 463.
Jensen, C., and Bajer, A. (1969). *J. Ultrastruct. Res.* **26**: 367.
Johnson, U. G., and Porter, K. R. (1968). *J. Cell Biol.* **38**: 403.
Jokelainen, P. T. (1967). *J. Ultrastruct. Res.* **19**: 19.
Jungers, V. (1931). *Cellule* **40**: 293.
Kalnins, V., and Porter, K. (1969). *Z. Zellforsch. Mikrosk. Anat.* **100**: 1.
Krisham, A., and Buck, R. C. (1965). *J. Ultrastruct. Res.* **13**: 444.
Kubai, D. F., and Ris, H. (1969). *J. Cell Biol.* **40**: 508.
Ledbetter, M. C. (1967). *Symp. Int. Soc. Cell Biol.* **6**: 55.
Ledbetter, M. C., and Porter, K. R. (1963). *J. Cell Biol.* **19**: 239.
Lettré, H., and Lettré, R. (1958). *Rev. Hematol.* **13**: 337.
Lima-de-Faria, A. (1958). *Int. Rev. Cytol.* **7**: 123.
Luykx, P. (1965a). *Exp. Cell Res.* **39**: 643.
Luykx, P. (1965b). *Exp. Cell Res.* **39**: 658.
Luykx, P. (1970). "Cellular Mechanisms of Chromosome Distribution." Int. Rev. Cytol; Suppl. 2. Academic Press, New York.
McIntosh, J. (1968). *J. Cell Biol.* **39**: 89a
McIntosh, J. R., Hepler, P. K., and Van Wie, D. G. (1969). *Nature (London)* **224**: 659.
Manton, I. (1964). *J. Roy. Microsc. Soc. Ser. B.* **83**: 471.
Marsland, D., and Landau, J. (1954). *J. Exp. Zool.* **125**: 507.
Marsland, D., Zimmerman, A. M., and Auclair, W. (1960). *Exp. Cell Res.* **21**: 179.
Maruyama, K. (1966). *Cytologia* **31**: 237.
Mazia, D. (1961). *In* "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 3, p. 77. Academic Press, New York.
Mercer, E. H., and Wolpert, L. (1957). *Exp. Cell Res.* **14**: 629.
Merriam, R. W. (1961). *Exp. Cell Res.* **22**: 93.
Metz, C. W. (1933). *Biol. Bull.* **64**: 333.
Mizukami, I., and Gall, J. G. (1966). *J. Cell Biol.* **29**: 97.
Molè-Bajer, J. (1967). *Chromosoma* **22**: 465.
Molè-Bajer, J. (1969). *Chromosoma* **26**: 427.

Molè-Bajer, J., and Bajer, A. (1968). *Cellule* **67**: 257.
Moor, H. (1966). *J. Cell Biol.* **29**: 153.
Moor, H. (1967). *Protoplasma* **64**: 89.
Moses, M. J., Wilson, M., and Wyrick, D. (1968). *J. Cell Biol.* **39**: 96a.
Nagai, R., and Rebhun, L. I. (1966). *J. Ultrastruct. Res.* **14**: 571.
Nakao, Y., Nakanishi, Y., and Wada, B. (1968). *Cytologia* **33**: 125.
Newcomb, E. H. (1969). *Annu. Rev. Plant Physiol.* **20**: 253.
Nicklas, R. B. (1967). *Chromosoma* **21**: 17.
Nicklas, R. B., and Koch, C. A. (1969). *J. Cell Biol.* **43**: 40.
Nicklas, R. B., and Staehly, C. A. (1967). *Chromosoma* **21**: 1.
O'Brien, T. P. (1967). *J. Cell Sci.* **2**: 557.
Östergren, G. (1949a). *Hereditas* **35**: 455.
Östergren, G. (1949b). *Hereditas* **35**: 525.
Östergren, G. (1950). *Hereditas* **36**: 1.
Östergren, G. (1951). *Hereditas* **37**: 85.
Östergren, G., Koopmans, A., and Reitalu, J. (1953). *Bot. Not.* p. 417.
Östergren, G., Molè-Bajer, J., and Bajer, A. (1960). *Ann. N.Y. Acad. Sci.* **90**: 381.
Pickett-Heaps, J. D. (1968). *Aust. J. Biol. Sci.* **21**: 255.
Pickett-Heaps, J. D. (1969a). *Aust. J. Biol. Sci.* **22**: 375.
Pickett-Heaps, J. D. (1969b). *J. Cell Sci.* **4**: 397.
Pickett-Heaps, J. D. (1969c). *J. Ultrastruct. Res.* **27**: 24.
Pickett-Heaps, J. D. (1969d). *Cytobios* **1**: 257.
Pickett-Heaps, J. D., and Fowke, L. C. (1969). *Aust. J. Biol. Sci.* **22**: 857.
Pickett-Heaps, J. D., and Northcote, D. H. (1966a). *J. Cell Sci.* **1**: 109.
Pickett-Heaps, J. D., and Northcote, D. H. (1966b). *J. Cell Sci.* **1**: 121.
Pollister, A. W., and Pollister, P. F. (1943). *Ann. N.Y. Acad. Sci.* **45**: 1.
Porter, K. R., and Machado, R. (1960). *J. Biophys. Biochem. Cytol.* **7**: 167.
Randall, J. T., and Jackson, S. F. (1958). *J. Biophys. Biochem. Cytol.* **4**: 807.
Rapport, R. (1969a). *J. Exp. Zool.* **171**: 59.
Rapport, R. (1969b). *Exp. Cell Res.* **56**: 87.
Rhoades, M. M., and Vilkomerson, H. (1942). *Proc. Nat. Acad. Sci. U.S.* **28**: 433.
Richards, B. M., and Bajer, A. (1961). *Exp. Cell Res.* **22**: 503.
Robbins, E., and Gonatas, N. K. (1964). *J. Cell Biol.* **21**: 429.
Robbins, E., and Jentzsch, G. (1969). *J. Cell Biol.* **40**: 678.
Roth, L. E., Wilson, H. J., and Chakraborty, J. (1966). *J. Ultrastruct. Res.* **14**: 460.
Sakai, A. (1968). *Cytologia* **33**: 318.
Schrader, F. (1953). "Mitosis, the Movements of Chromosomes in Cell Division," 2nd Ed. Columbia Univ. Press, New York.
Schroeder, T. E. (1968). *Exp. Cell Res.* **53**: 272.
Seto, T., Kezer, J., and Pomerat, C. (1969). *Z. Zellforsch. Mikrosk. Anat.* **94**: 407.
Sinnott, E. W., and Bloch, R. (1941). *Amer. J. Bot.* **28**: 225.
Sorokin, S. P. (1968). *J. Cell Sci.* **3**: 207.
Stephens, R. (1970). *In* "Biological Macromolecules" (S. N. Timasheff and G. D. Fasman, eds.), Vol. 4. Dekker, New York. In press.
Stich, H. (1954). *Chromosoma* **6**: 199.
Stubblefield, E., and Brinkley, B. R. (1968). *Symp. Int. Soc. Cell Biol.* **6**: 175.
Subirana, J. A. (1968). *J. Theor. Biol.* **20**: 117.
Szollosi, D. (1968). *J. Cell Biol.* **39**: 133a.

Szollosi, D. (1970). *J. Cell Biol.* **44**: 192.
Tilney, L. G., and Marsland, D. (1969). *J. Cell Biol.* **42**: 170.
Tucker, J. B. (1968). *J. Cell Sci.* 3: 493.
Wada, B. (1966). *Cytologia* **30** (Suppl.): 1.
Wagenaar, T. B. (1961). *Can. J. Bot.* 39: 81.
Weinstein, R. S., and Hebert, R. R. (1964). *J. Cell Biol.* **23**: 101a.
Wilson, E. B. (1928). "The Cell in Development and Heredity." Macmillan, New York.
Wilson, H. J. (1968). *Planta* **78**: 379.
Wilson, H. J. (1969). *J. Cell Biol.* **40**: 854.
Wohlman, A., and Allen, R. D. (1968). *J. Cell Sci.* 3: 105.
Zimmerman, A. M., and Marsland, D. (1956). *Biol. Bull.* **111**: 317.
Zirkle, R. E. (1957). *Advan. Biol. Med. Phys.* **5**: 103.

A UNIT MITOCHONDRION: DNA CONTENT AND RESPONSE TO X-IRRADIATION*

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I. Introduction

Noyes and Smith (1959) were the first to report an early, dramatic change in the mitochondria of rat livers after irradiation with 1000 roentgens. The mass of individual mitochondria isolated by differential centrifugation was almost doubled, though small mitochondria appeared to be preferentially fragmented. At the same time, the total nitrogen of the liver as a whole remained essentially unchanged. Values for mitochondrial parameters returned to normal only after a few hours of recovery. These interesting observations followed earlier comparative studies by Smith (1956) and employed techniques developed by him. His methods involved isolating the mitochondria and counting them under the phase microscope, with subsequent biochemical determinations of protein nitrogen content.

Since 1956, the author and his collaborators have developed a practical method and instrumentation for the sensitive determination of dry mass with the electron microscope (Bahr *et al.*, 1956, 1961; Zeitler and Bahr, 1957, 1962, 1965; Bahr, 1957; Bahr and Zeitler, 1965a). This makes it possible to determine the masses of small biological objects weighing from 10^{-11} gm to an extreme of 10^{-18} gm, i.e., from whole cells to macromolecules. The theory and applications of the technique were thoroughly reviewed by Bahr and Zeitler (1965a).

In view of the great interest in early effects of ionizing radiation on nonnuclear, cytoplasmic components, our group has repeated the experiments of Noyes and Smith (1959), using the more precise techniques of quantitative electron microscopy. Our primary objectives were as follows:

1. To study postirradiation changes in dry mass of individual mitochondria.

2. To compare mitochondria from the livers of rats exposed to whole-body radiation with those from livers shielded from direct radiation, or irradiated separately, in order to determine whether the mitochondrial and cytoplasmic changes result from processes outside the liver or constitute a direct response to irradiation by the liver itself.

3. To carry out thorough morphological follow-up of cellular events, supplementing the quantitative electron microscope analysis by using both thin section electron microscopy and light microscopy.

Our first experiments confirmed that liver mitochondria undergo considerable changes as a consequence of irradiation. It also became apparent that determination of mitochondrial dry mass alone, although providing unique information, permitted only limited interpretation; it

was necessary to obtain data on mitochondrial volumes and concentrations of mitochondrial solids as well. Consequently, a scheme of multiple technical approaches to mitochondrial mass, volume, concentration, and numbers was devised, and the methodological requirements were studied in detail by Glas and Bahr (1966).

In partial confirmation of the earlier work by Noyes and Smith (1959), we found that the average dry masses of mitochondria did indeed increase after irradiation (Bahr and Glas, 1964). Our statistical data, based on numerous measurements of *individual* mitochondria, suggested that the irradiation effect was proportional to mitochondrial dry mass, or to some factor that is itself proportional to dry mass. Mitochondrial DNA was considered a likely target because cellular radiosensitivity had been found by Sparrow and Miksche (1961) to be closely correlated with nuclear volume and subsequently with chromosomal mass and DNA mass (Kaplan and Moses, 1964; Sparrow *et al.*, 1967; Baetcke *et al.*, 1967).

Our most recent experiments do reveal a close proportionality of mitochondrial DNA to mitochondrial dry mass, and they suggest that the smallest mitochondria may contain only one circular DNA molecule, constituting a hypothetical *unit mitochondrion*—the building block of all rat liver mitochondria and the target volume in the irradiation study (Bahr, 1969).

II. Materials and Methods

A. Animals

We used Osborne-Mendel rats weighing from 200 to 250 gm from a colony kept at the Armed Forces Institute of Pathology under veterinary health control. A commercial food preparation and water were provided *ad libitum*. The animals were killed by stunning and bleeding at comparable times in the morning without previous fasting.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council.

B. Irradiation

For the total-body irradiation of the animals, we used a Maxitron X-ray source at 200 kV filtered through 6 mm of copper. The rats were

placed in constrainers manufactured from thin acrylic plastic in such a way that irradiation penetrated the animal in the ventrodorsal direction; slight pressure flattened the abdomen somewhat but did not interfere with breathing. Continuously monitored radiation at a dose of 1000 r was administered in 4 minutes. In a second series of exposures the liver area of each animal's abdomen was shielded with lead foil 3 mm thick. In a third series only the liver area was exposed, while the rest of the rat was shielded with a 3-mm thickness of lead foil. Both the fitting of the shield protecting the liver area and the opening for exposing the liver area alone were approximate, inasmuch as individual liver size and topography were not precisely known. Of course, other organs were also in the path of the X-ray beam. Animals were sacrificed at 0.5, 1, 3, 6, 12, 15, 24, 48, and 72 hours after irradiation, and their livers were removed immediately.

C. Electron Microscopy of Thin Sections

Pieces of normal and irradiated rat liver were submersed in ice-cold Dalton's chrome—osmium fixative for electron microscopy and further dissected into small cubes under the fixative. These cubes were dehydrated in a graded ethanol series and embedded in Epon 812 according to conventional techniques. Sections from all blocks were photographed with a Siemens electron microscope at one carefully controlled magnification (Bahr and Zeitler, 1965b), and subsequent stereological analysis of mitochondrial populations was carried out according to the techniques of Loud and co-workers (1965; Loud, 1962) and Weibel *et al.* (1966). Other sections were studied for detail at different magnifications.

D. Isolation of Mitochondria

Glas and Bahr (1966) published a separate report on the isolation of mitochondria for the purposes of this study. In brief, the method requires 40 ml of 0.44 *M* sucrose for the homogenization of 15 gm of liver tissue (usually the pooled livers of two animals), followed by centrifugations at 1200 *g*, three times; at 1700 *g*, one time; and finally, at 8500 *g*, two times (10 minutes each). The method yields mitochondrial preparations of singular purity; Figs. 1 and 2 are representative electron micrographs illustrating such a preparation. We monitored the purity of the mitochondrial suspensions not only by thin sectioning pellets according to the regimen described in the preceding section, but also with unsectioned samples on Formvar-coated specimen grids.

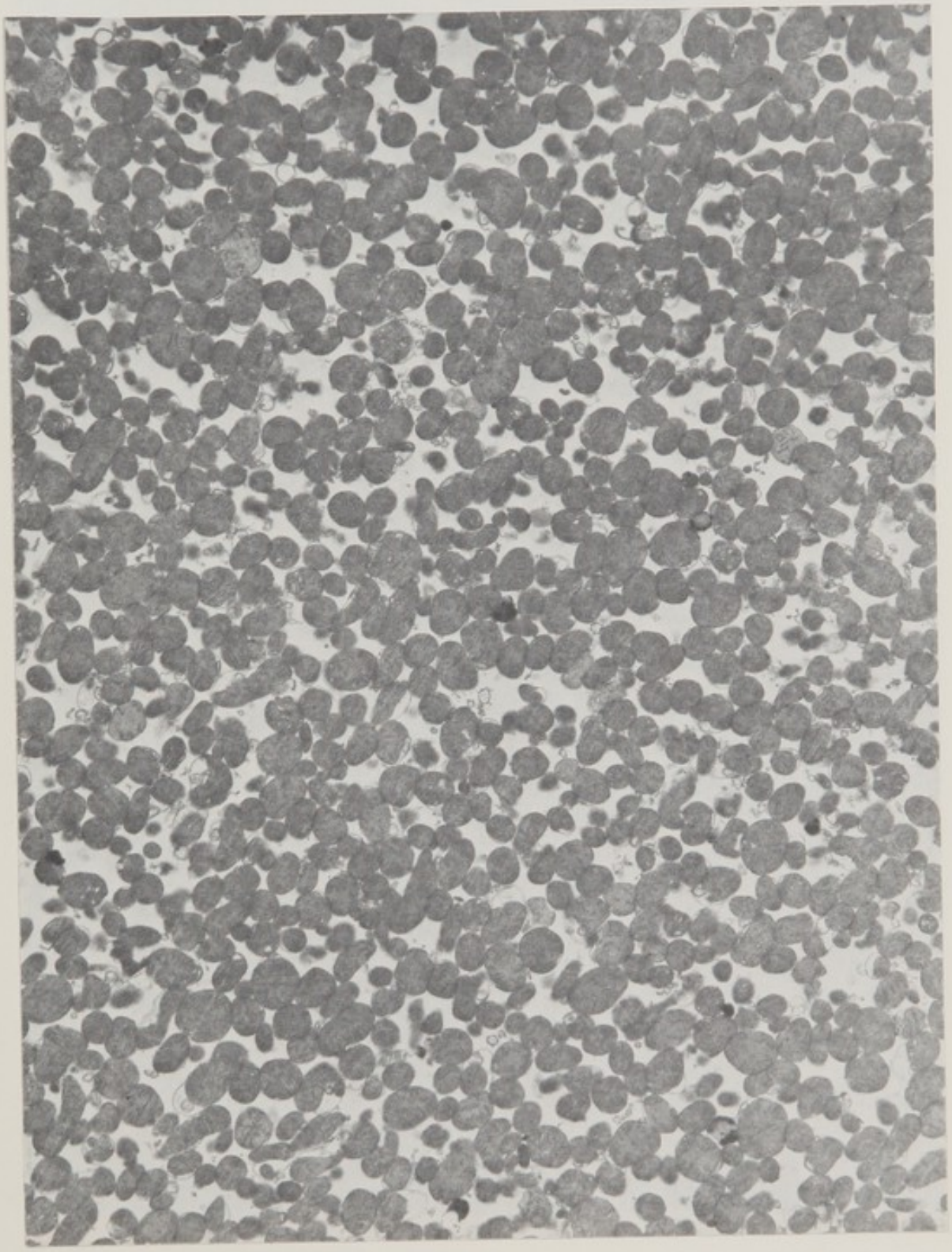

FIG. 1. A low-power electron micrograph of thin-sectioned mitochondria from a pellet 6 hours postirradiation. The frequency of spherical versus elongated mitochondrial profiles is normal. However, there are 3% "light mitochondria." In about 10% of the profiles there is sequestration of membrane material inside the outer membrane, as detailed in Fig. 4. 5300×. AFIP neg. 70-2035-1.

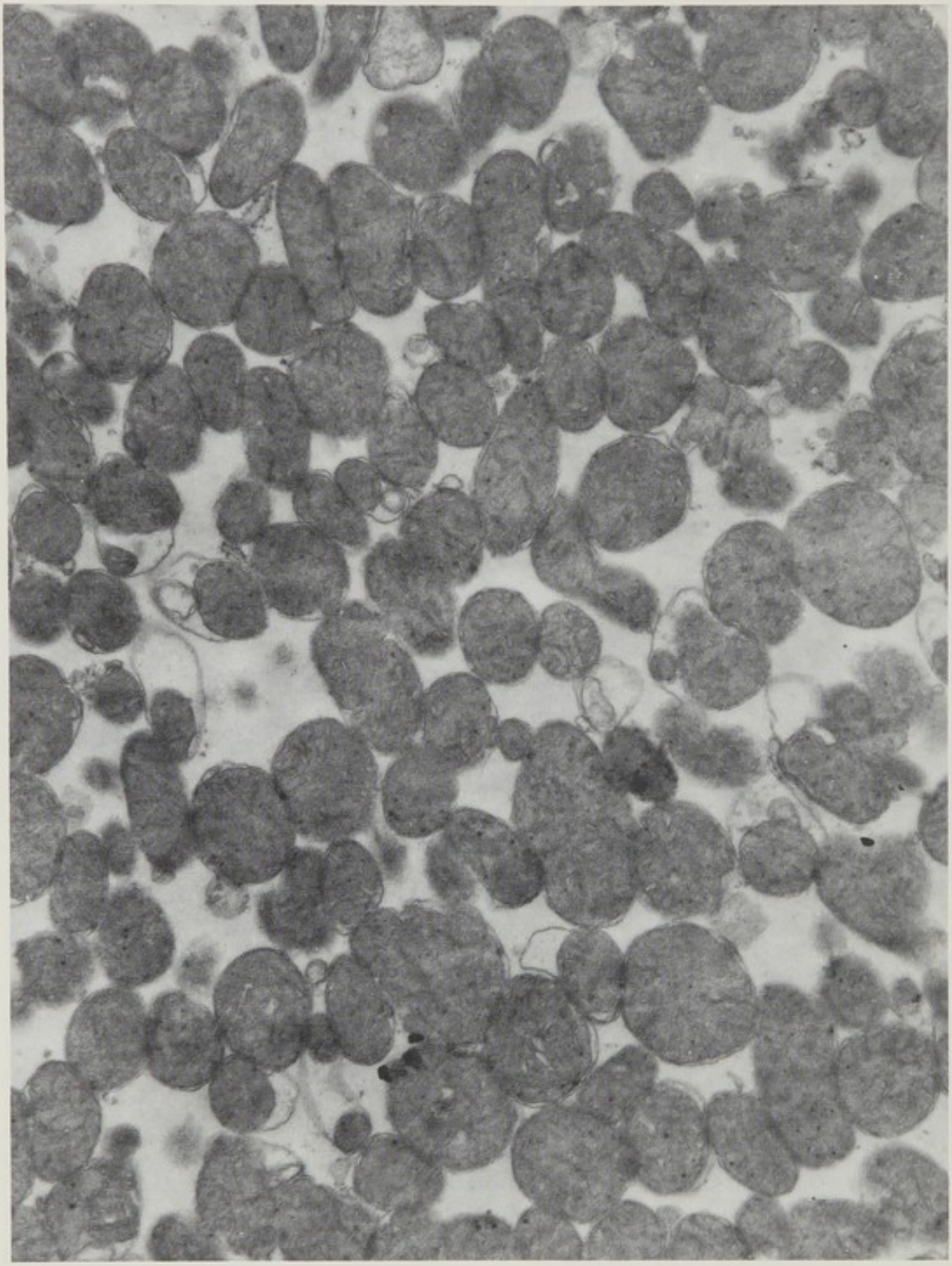

FIG. 2. A higher magnification electron micrograph from the same thin-sectioned pellet 6 hours after irradiation. Note the normal appearance of mitochondria in standard configurations. The number of mitochondrial granules is also normal, but there is a significant sequestration of membrane material inside the outer membrane, together with loosening of the membrane's normal close fit to the contours of the mitochondrial matrix. 12,800×. AFIP neg. 70-2035-2.

The degree of admixture of nonmitochondrial cell fragments is most easily assessed by whole mount electron microscopy (Glas and Bahr, 1966).

E. Quantitative Determination of Mitochondrial Dry Mass

Immediately after the last centrifugation, the pellet was suspended in 5 ml of 0.44 *M* sucrose, and 10 ml of 10% formalin at pH 7.2 was added and mixed. The specimen was kept in the fixative for 20 minutes. Droplets of the fixed mitochondrial suspension were then placed on Formvar-coated grids and after standing 2 to 5 minutes were blotted from the edge with filter paper. After further drying, a gentle rinse of distilled water for half a minute removed any remaining sucrose.

The mitochondria were photographed with a Siemens electron microscope under controlled conditions of accelerating voltage, contrast aperture, electron beam current, exposure, and magnification; these conditions were chosen to assure proportionality between the dry mass of the object and the transmission of the image of the object in the electron micrograph. All micrographs were processed in temperature-controlled photochemicals side by side with electron micrographs of dry mass standards (polystyrene spheres) and a magnification standard. By comparing the light transmissions of individual mitochondrial images with those in electron micrographs of polystyrene spheres of known mass photographed under identical conditions, it was possible to calculate the absolute dry mass (weight) of each mitochondrion. Figure 3 shows a curve relating the weight of polystyrene spheres to transmission $(T - T_0)$, where T is the integrated (averaged) transmission over the object and T_0 is the transmission over the immediate background of the object. An accurate, fast, and simple means to determine these transmissions is provided by the integrating photometer IPM-2 manufactured by Carl Zeiss, Oberkochen.

The technique for dry mass determination by electron microscopy was developed by the author and Dr. E. Zeitler; applications and theoretical considerations are described in several published papers (see especially Bahr and Zeitler, 1965c).

F. Zonal Centrifugation

Six mitochondrial pellets were prepared from 12 rat livers, according to the procedure for isolation of mitochondria outlined above. These pure preparations were pooled and suspended to make a volume of 100 ml in 0.44 *M* sucrose, and within 5 minutes a suspension was pumped

through the rotor axis onto a gradient of 25 to 45% sucrose spinning at 5000 rpm in the zonal rotor BIV of the L4 zonal centrifuge (Beckman Instrument Co.). Tubing and injector pathways were rinsed with 40 ml of 0.44 *M* sucrose, which also served as an overlay. Without change of centrifuge speed, the rotor was spun for 1 hour, after which it was emptied by introducing a solution of 50% sucrose at the periphery of the rotor. The effluent was divided into fractions of 30 ml, after discarding the first 150 ml. Fractions 1–8 contained the small mitochondria,

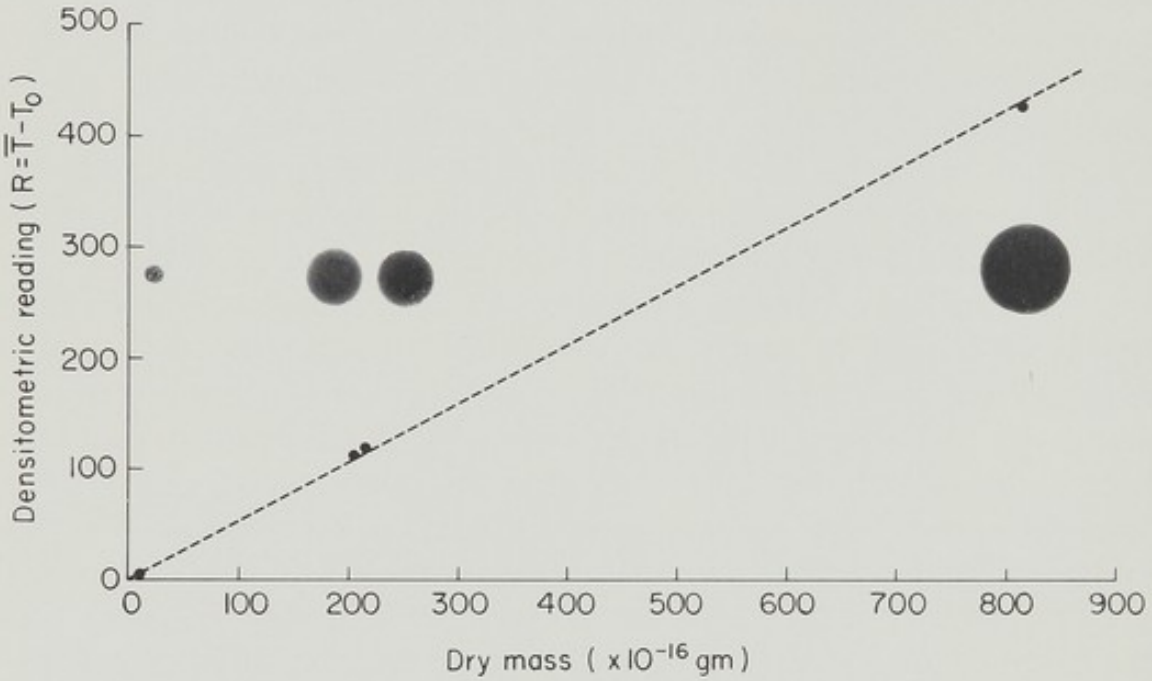

FIG. 3. A calibration curve for quantitative determination of dry mass with the electron microscope. The difference between the integrated transmission of polystyrene latex spheres (10029-31,32,33) and their backgrounds (densitometric reading $R = \bar{T} - T_0$) is proportional to the absolute mass of each sphere. The latter value is calculated from the diameter of the sphere giving its volume, times the specific gravity of polystyrene. AFIP neg. 70-2035-3.

10–17 the medium, and 19–27 the large. Centrifugation at 20,000 rpm in rotor 30 for 5 minutes pelleted the mitochondria, which were pooled for each group by resuspending them in 10 ml of 0.44 *M* sucrose. A solution of 0.1 mg DNase/ml + 0.005 *M* $MgSO_4$ in 0.25 *M* sucrose was added to raise the volume to 35 ml. The mitochondrial suspensions were then incubated at 37°C for 15 minutes.

G. DNA Determination

DNase-treated samples were cooled in ice, centrifuged at 20,000 rpm for 10 minutes, and washed once in 0.44 *M* sucrose. After the supernatant

sucrose had been poured off, the centrifuge tubes were inverted over filter paper and drained for about 10 minutes. Finally, the exposed wall on the inside of each tube was wiped dry with swabs, and the tube was weighed to determine the fresh weight of the pellet. The pellets were then resuspended in 10% trichloroacetic acid 1:1 at 90°C for 15 minutes. Two successive extractions followed, the supernatants being pooled after centrifugation at 20,000 rpm in rotor 30. All subsequent steps followed the diphenylamine method used by Burton (1956).

H. Calculation of Size

Because it was the experimental aim to elucidate quantitatively the relationship of mitochondrial DNA to mitochondrial mass, determination of pellet dry mass was not a prerequisite, rather the *wet* pellet weight was considered an adequate correlate quantity. It was assumed that intermitochondrial space was 20% of the pellet and contained essentially water.

Mitochondrial wet weight includes close to 25% solids (Glas and Bahr, 1966). The total amount of solids was calculated for each of the three pellets—small, medium, and large—and divided by the average mitochondrial dry mass found for that pellet by quantitative dry mass determinations in the electron microscope. In this way the estimated number of mitochondria in each pellet could be calculated.

Finally, the absolute amount of DNA found for each of the three mitochondrial pellets prepared in an experiment was divided by the number of mitochondria, giving the absolute DNA content for an average mitochondrion in that pellet.

III. Results

A. Morphological Effects

As judged qualitatively, no single morphological change distinguished mitochondria pelleted from irradiated tissue compared with unambiguously normal mitochondria. Even though several changes in cytoplasmic structures appeared to be occurring more frequently in irradiated hepatocytes than in nonirradiated tissues, these were inaccessible for quantitative evaluation. Nevertheless, specimens prepared from livers irradiated while the remainder of the rat's body was shielded with lead were generally comparable to specimens subjected to total-body irradia-

tion. By contrast, liver sections obtained from rats in which the liver had been shielded were fully compatible with normal tissue.

Because the normal structural variability of rat liver hepatocytes requires lengthy statistical analysis (e.g., sectional or modal analysis, planimetry), the ill-defined changes in irradiated specimens could not be established from thin sections without prohibitive effort involving cumbersome and time-consuming measurement procedures. Blind tests demonstrated that it is impossible to distinguish with certainty irradiated from nonirradiated tissue, to say nothing of differentiating postirradiation recovery periods. For this reason, attempts to assess subtle quantitative changes in the usual thin-sectioned appearance of irradiated livers were finally abandoned.

Some morphological features remained, which could be found in normal tissue, but which impressed several observers by their relatively greater abundance or prominence in liver sections from irradiated rats. The following features may represent early morphological evidence of irradiation damage to hepatocytes: (1) A derangement of nuclear content is present in some cells, producing slight margination of chromatin and aggregation of granules. (2) Part of the nucleolar structure condenses (becoming more electron dense). (3) Nuclear pores become more prominent. (4) The outer membrane of the nuclear envelope, a part of the endoplasmic reticulum, lifts off while the perinuclear space fills with a clear liquid; this alteration of the nuclear envelope was found only in cells already having a general distension of vesicles of the endoplasmic reticulum. Although engorgement of the smooth and rough endoplasmic reticulum can also be found in some nonirradiated hepatocytes, this is more prominent in irradiated liver and becomes more pronounced as time elapses after irradiation. The rough endoplasmic reticulum leads in this respect, but it has largely returned to normal within 6 hours after irradiation. By contrast, the smooth endoplasmic reticulum continues to distend, and parts of it disrupt into assemblies of small vesicles at about 48 hours and thereafter (Fig. 4). (5) Glycogen depletion starts at 1 hour and is complete in 6 to 15 hours postirradiation. It is accompanied by an increase and enlargement of droplets of neutral fat. (6) The Golgi apparatus distends during the first 6 hours, but appears normal later on. (7) The presence of a moderately electron dense mass in the center of a mitochondrion is a rare finding in normal hepatocytes, but in irradiated tissue such densities are found in three or more mitochondria per cell in each thin section. No cristae transect such densities; they are most prominent 1 hour after the irradiation insult, but seem to be present at all subsequent times.

Many mitochondria isolated from animals 1 to 15 hours postirradiation

showed sequestration of membraneous material from the inner mitochondrial membrane and, at times, a small dense body (Fig. 5). The number of dense mitochondrial granules stayed fairly constant throughout the postirradiation period studied, but there was a slow decrease of long mitochondrial profiles, leaving only a very few in pellets prepared 48 or 72 hours postirradiation. As time elapsed after irradiation, the number of swollen and fragile mitochondria increased in the pellets, though they never exceeded 12% of the visible profiles. There was also an apparent increase in the number of mitochondria up to 6 hours postirradiation. At 15 hours and later small myelin figures, lipid droplets, lysosomes, and larger groups of neutral fat globules appeared in the cytoplasm; at this time, we probably were seeing the already incipient

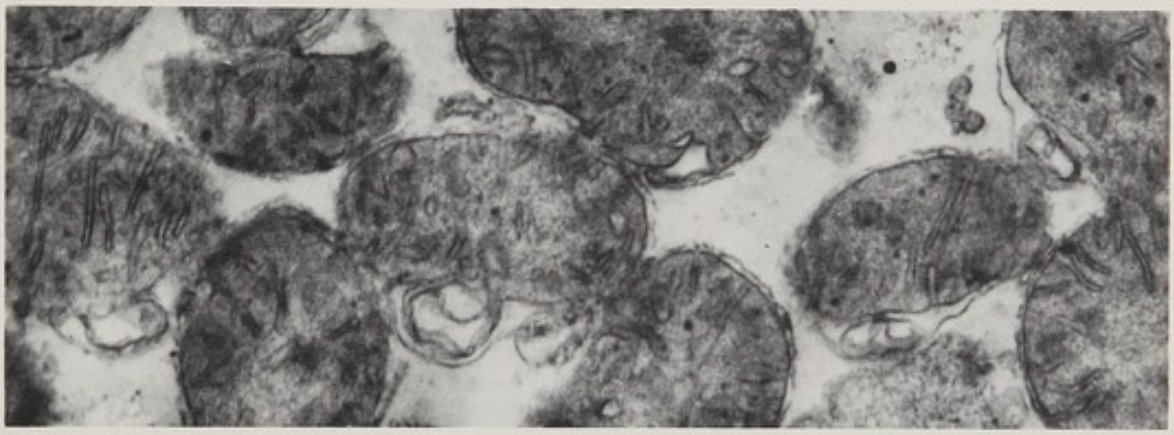

FIG. 4. A thin-sectioned hepatocyte, showing distension of rough and smooth endoplasmic reticulum at 3 to 15 hours postirradiation. The mitochondria appear normal. 15,400×. AFIP neg. 70-2035-5.

consequences of a bone marrow irradiation syndrome, as well as the consequences of inanition in the irradiated animals.

Quantitative evaluation of mitochondrial profiles in thin sections of liver cells studied by planimetry of enlarged prints gave no conclusive data. Figure 6 shows the results of such measurements, and represents the mean of means for extensive series based on at least five irradiation experiments each. There was a rapid rise of the grand mean of the cross-sectional area within 1 hour after irradiation, and a subsequent drop of area (viz., volume) to almost normal levels at 6 hours postirradiation. However, these observations were largely invalidated by the large variation of the means for individual experiments.

In contrast to the measurements on thin-sectioned liver cells, we found a slow but distinct rise in the mean cross-sectional areas of isolated mitochondria in sectioned pellets (Fig. 6). There was little variance of the means; the increase peaking at 6 hours postirradiation represented

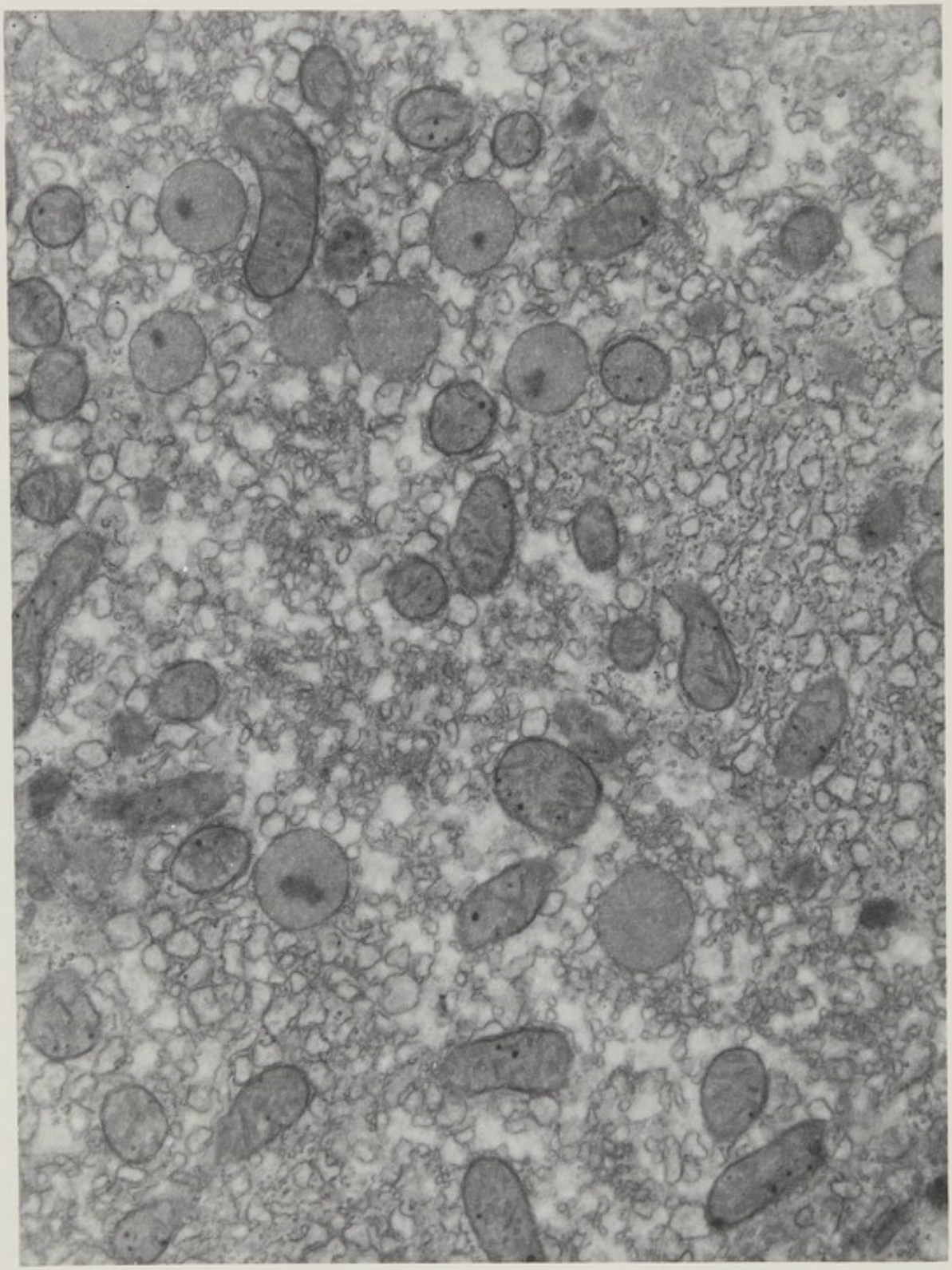

FIG. 5. A detail showing several examples of membrane sequestration. The morphology suggests that they arise from the inner mitochondrial membrane. 23,000×. AFIP neg. 70-2035-4.

a 20% rise, but this is a small increase for cross-sectional area. We concluded that visual inspection of electron micrographs alone could not reveal which specimens had been irradiated and which had not. The

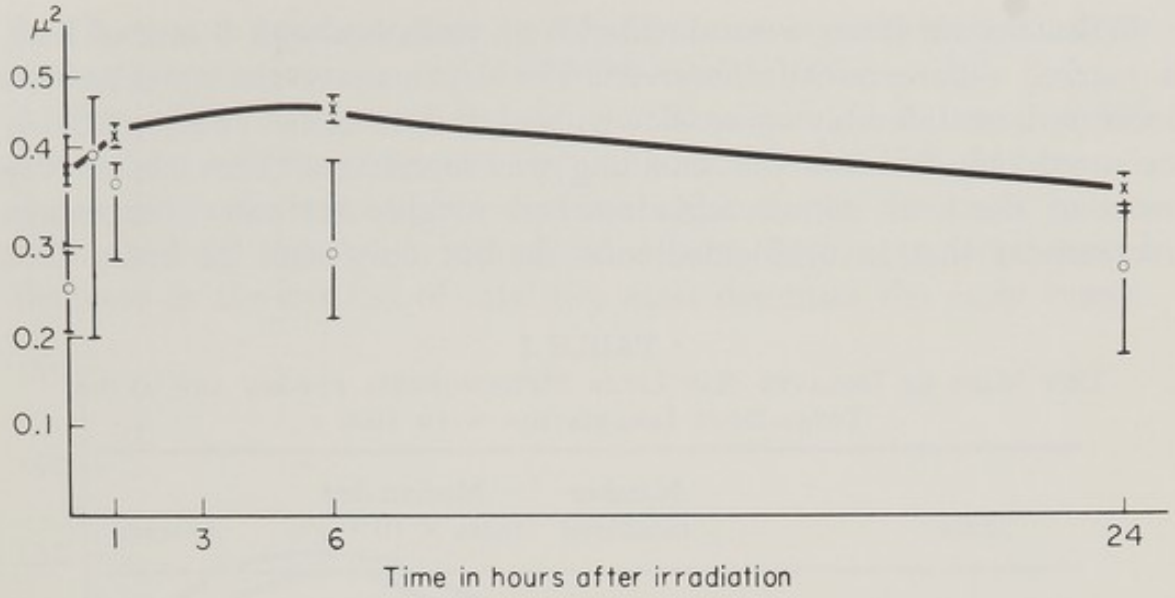

FIG. 6. Changes in the mean area (μ^2) of mitochondrial profiles from rat liver in thin-sectioned tissue (○) and in pellets (×) following X-irradiation with 1000 r. The bars show the spread of mean values obtained from various thin-sectioned blocks and experiments. AFIP neg. 70-2035-6.

task of the investigator was further complicated by having to draw conclusions from a range of sizes in cross-sectional profiles produced by a random plane of sectioning.

B. The Dry Mass of Individual Mitochondria

All quantitative determinations of mitochondrial properties following irradiation were done in collaboration with Dr. Ulla Glas while she was a visiting scientist in our biophysics laboratories. Volume, nitrogen content, and concentration of solids in mitochondria were determined individually and directly according to the methods discussed in detail by Glas and Bahr (1966; see also Bahr and Glas, 1964).

A rapid increase was found in the median *dry mass* of populations of mitochondria isolated at various times after total-body irradiation (Table I). The dry mass curve in Fig. 7 shows the rise to be about 30% over normal values. After reaching a maximum at 6 hours postirradiation, the dry masses dropped but remained slightly elevated over normal values. Concomitantly there was an increase in *volume* that exceeded the rise in dry mass, thereby indicating a slight degree of swelling. Furthermore, the nitrogen content per mitochondrion accompanied the increase in dry mass as evidence that the accumulating solids in the mitochondrion did not consist of inorganic substances. The disparity between increases in dry mass and in volume produced a decrease in the concentration of solids per mitochondrion (nitrogen/μ^3; see Fig. 7).

When the rat livers were shielded from radiation with 3 mm of lead, a marked difference was observed. The dry mass of the mitochondria increased considerably more slowly, and this minimal effect probably occurred only because the shielding was insufficient. The median dry mass of the liver mitochondria reached roughly the same percentage increase as that in unshielded animals, but only after 24 hours (see

TABLE I

DRY MASS OF ISOLATED RAT LIVER MITOCHONDRIA BEFORE AND AFTER TOTAL-BODY IRRADIATION WITH 1000 r

Time	Number measured	Median dry mass $\times 10^{-14}$ gm	Average
Preirradiation	487	11.57	11.42
	289	12.05	
	420	11.79	
	194	12.28	
Postirradiation			
30 minutes	682	12.84	14.20
	445	14.97	
	542	14.98	
1 hour	466	14.74	14.77
	382	14.81	
3 hours	266	16.20	15.01
	513	14.28	
	354	14.83	
6 hours	329	20.20	17.77
	471	15.35	
15 hours	426	13.51	12.32
	226	11.13	
24 hours	549	13.96	13.08
	305	12.21	
48 hours	224	12.67	13.39
	153	13.38	
	226	14.13	
72 hours	482	14.49	14.49

Fig. 8 and Table II). There was also little decrease in the concentration of solids in these experiments (Fig. 8). If instead the animal itself was shielded and the liver alone was exposed to X-rays, the increase in the dry mass of individual mitochondria appeared to be faster and possibly more extensive than in total-body irradiation (Fig. 8), without significantly decreasing the concentration of solids beyond levels already observed in total-body irradiation.

We conclude from these data the following facts:

1. There is a rapid increase in the dry mass of individual rat liver mitochondria as a consequence of irradiation with 1000 r.

2. The effect is transient, reaching a maximum at 6 hours postirradiation and returning to values close to normal within 15 to 24 hours.

3. Swelling of mitochondria occurs, but it is not the main reason for the observed increase in mitochondrial size. Rather, an increase and a decrease in the median of total *dry mass* dominate the early events.

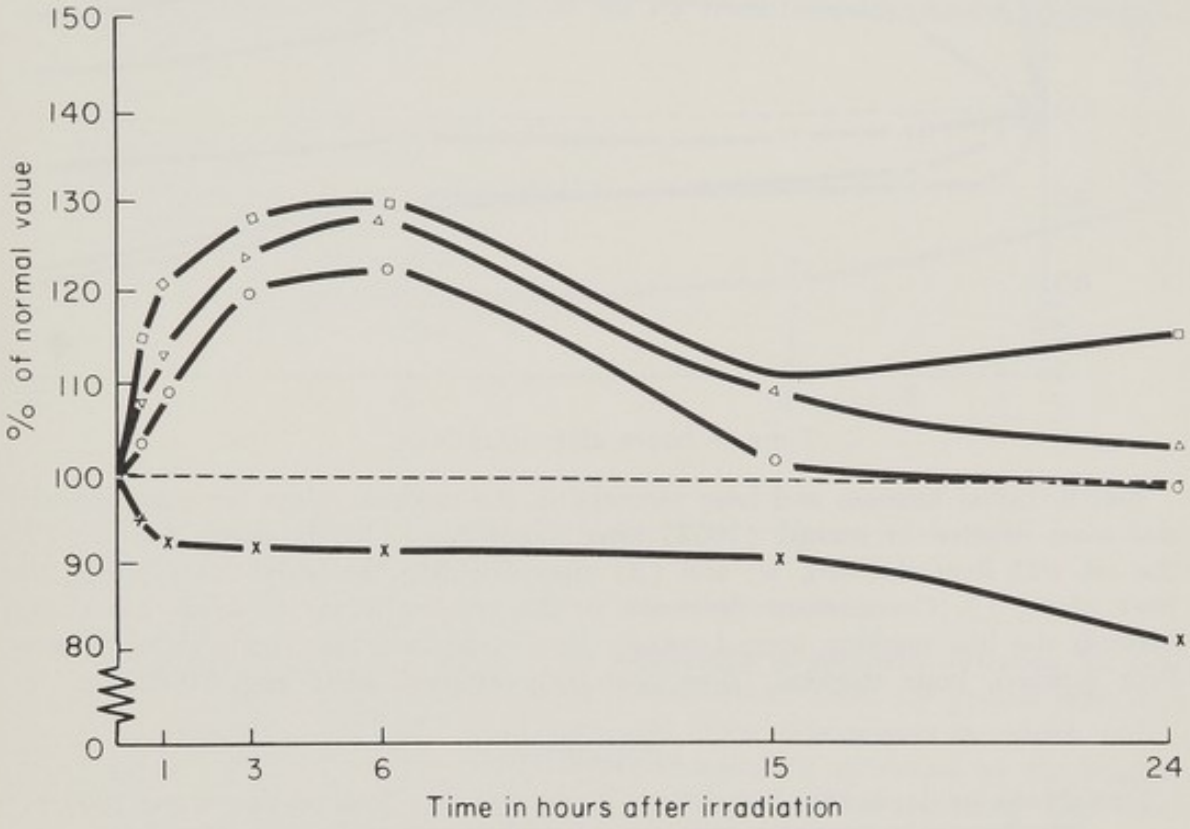

FIG. 7. Initial increase and later recovery in the median values for mitochondrial dry mass relative to normal (100%) after total-body irradiation. △ = dry mass determined by quantitative electron microscopy; □ = mitochondrial volume determined with the aid of a Coulter counter or from mitocrits; ○ = protein nitrogen per mitochondrion; × = concentration of solids (for methods, see Glas and Bahr, 1966). AFIP neg. 70-2035-7.

4. The early effect of irradiation on mitochondria is direct and apparently not mediated by other radiosensitive organs, such as the intestine.

So far only median dry mass values for entire mitochondrial populations have been considered. When the response of the population in terms of its distribution into mitochondrial sizes or dry masses was investigated, we found first that two types of mitochondria were distinguishable in transmission electron micrographs of intact mitochondria, one being more electron transparent than the other. When the weights

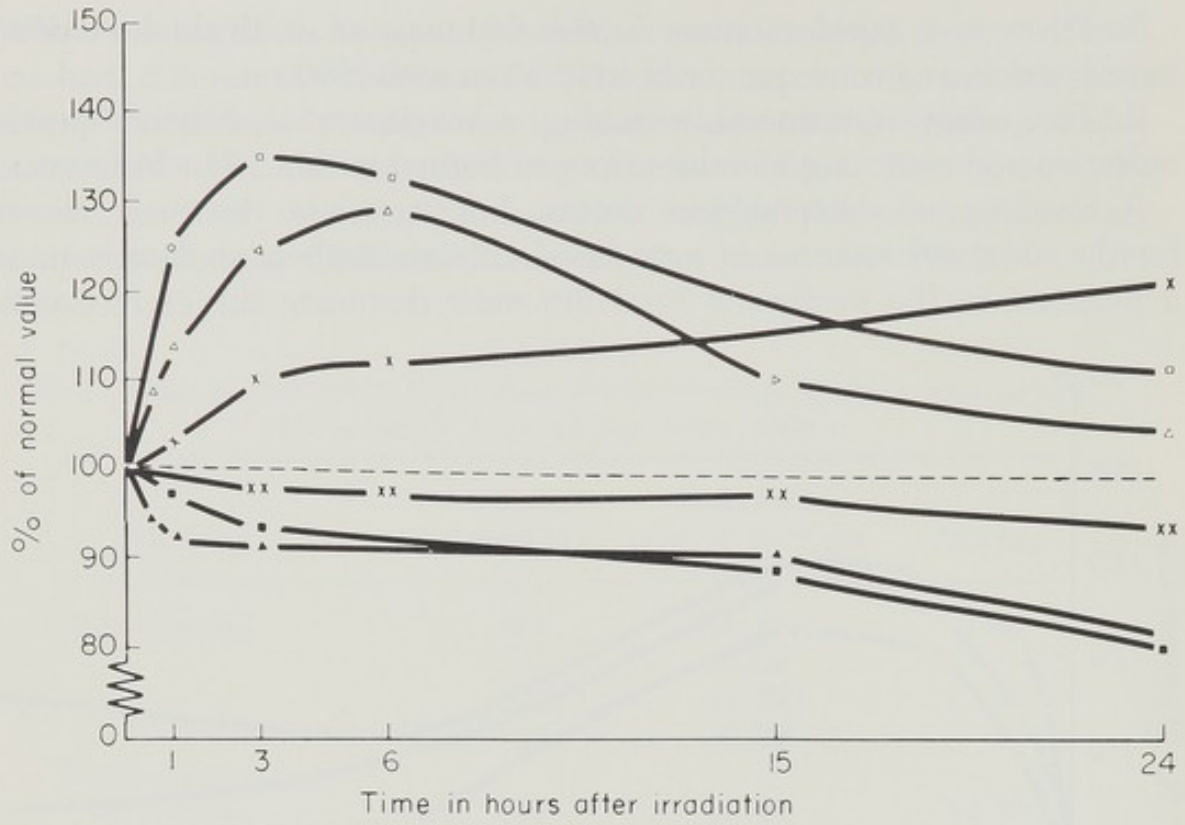

FIG. 8. Initial increase and later recovery in the median values for mitochondrial dry mass relative to normal (100%) after irradiating: (1) the entire rat, △; (2) the rat with liver shielded, ×; and (3) after shielding the body and exposing the liver alone, □. Concomitant decreases in the concentrations of solids are shown beneath the line marking normal values. ×× = shielded liver, body exposed; ■ = liver exposed, body shielded; ▲ = total body exposed. AFIP neg. 70-2035-8.

TABLE II

DRY MASS OF ISOLATED RAT LIVER MITOCHONDRIA IRRADIATED WITH 1000 r

Time postirradiation (hours)	Number measured	Median dry mass	Average
	Liver shielded, body exposed		
1	205	11.94	11.92
	341	11.90	
3	384	12.12	12.12
6	379	12.80	12.87
	344	12.94	
24	291	15.03	15.03
	Liver exposed, body shielded		
1	315	14.46	14.46
3	304	15.60	15.60
6	388	19.29	19.29
24	330	13.79	13.79

of these two populations were determined separately, the lighter population was found to represent about 17% of the total number and had a median mass only one fifth to one fourth of that characterizing the major population. These objects were not fragments, but were comparable to the heavier population in size and shape.

Second, we noted that the spread (log σ of dry mass) for the major population remained remarkably constant up to 6 hours postirradiation, indicating that the dry mass of all its members increased in proportion

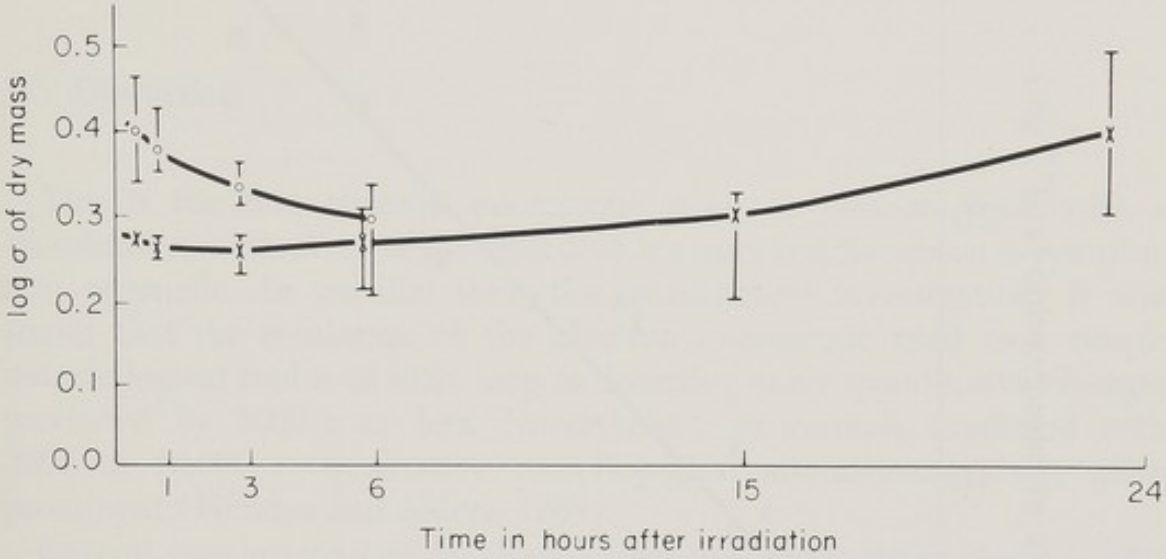

FIG. 9. The course of change in the logarithms of standard deviation for median values of mitochondrial dry mass after X-irradiation. Data are pooled from experiments involving total-body irradiation and from experiments in which only the liver was exposed. A subfraction of light mitochondria produced by the irradiation is indicated by ○, while the crosses (×) denote normal-appearing mitochondria. Bars indicate the *spread* of standard deviations for tissues after irradiation. The mean of the standard deviations stays remarkably constant up to 15 hours, in spite of some increasing variability. AFIP neg. 70-2035-9.

to the respective dry masses at the time of irradiation (Fig. 9). The spread of the lighter population, at the earliest times after irradiation, was considerably larger than that of the major population, but decreased with time (Fig. 9). At 15 and 24 hours the two populations could no longer be clearly distinguished in the electron micrographs, so that the two populations appeared as one, which had an ever-increasing spread of dry mass values. By contrast, normal, nonirradiated mitochondria constitute only a single population.

C. Correlation of Dry Mass with DNA Content

Zonal centrifugation of pure mitochondrial populations produced an effective separation of mitochondria into groups of different sizes. Our

technique was a hybrid operation in which the effects of both a density gradient and the differential sedimentation velocities of particles of different sizes reinforced each other. Because of slight variations in loading time, gradient temperature and therefore viscosity, as well as some variations in the amount of mitochondria separated, we found some differences in dry mass and size groupings for each separation run. Last

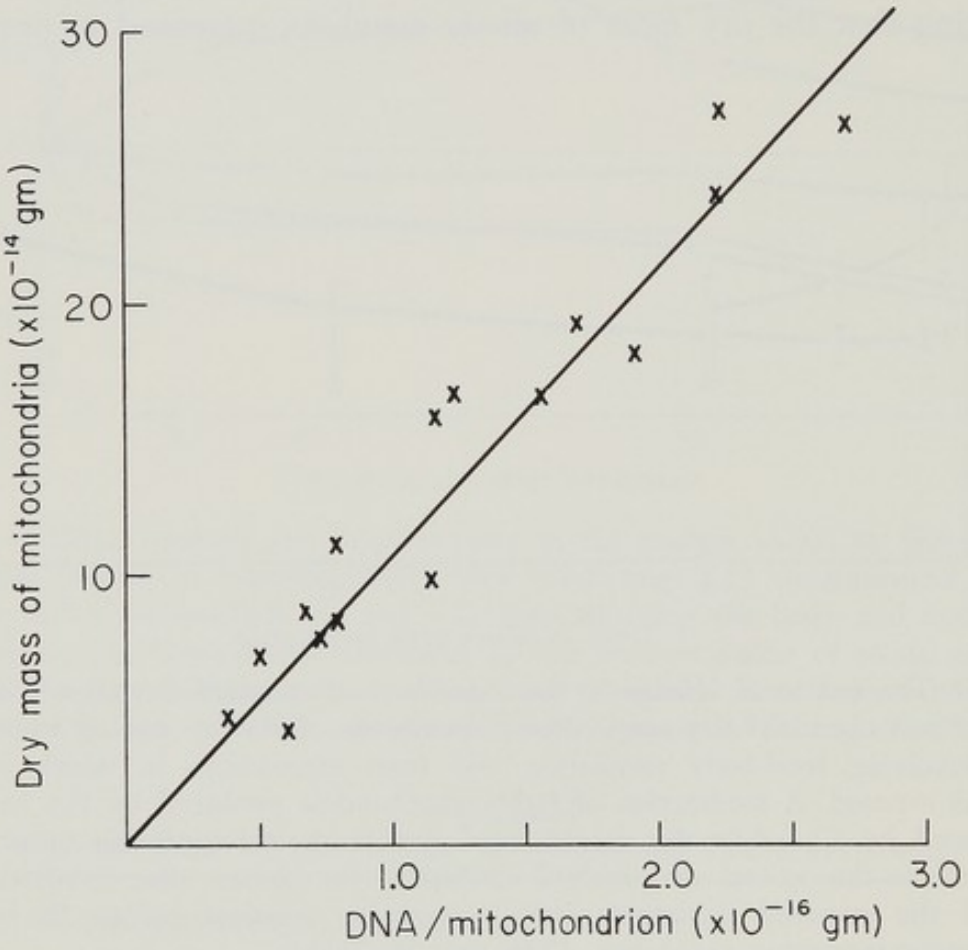

FIG. 10. The mean for mitochondrial dry mass in a given subfraction of purified mitochondria appears on the ordinate, while the DNA content for an average mitochondrion in the same subfraction is shown on the abscissa. DNA content is closely proportional to mitochondrial dry mass. In rat liver, the slope of the regression line is 0.9 μg M-DNA/mg of dry mass. AFIP neg. 70-2035-10.

but not least, some experimental parameters such as quantities of "cushion" and "overlay" were deliberately changed in the course of the series to search for more effective conditions for separation. Nevertheless, a separation of three fractions—large, medium, and small—could be obtained by arbitrarily cutting the continuum of fractions into three approximately equal portions. The average dry mass was determined for each of the three fractions by quantitative electron microscopy and compared with the average DNA content per particle. Figure 10 illustrates

the calculated regression line for all values, together with individual correlations. A gross division of the data into heavy, medium, and light samples is apparent, but it is least distinct for the smallest mitochondria. The regression curve intercepts very close to the origin (y intercept $= 0.09 \times 10^{-14}$ gm), which means that all of the dry mass of the mitochondrion is correlated with the presence and amount of DNA. A proportionality of 0.9 μg DNA/mg dry mass is represented by the slope of the regression line.

IV. Discussion

This is the first electron microscope study to concern itself with a quantitative evaluation of the effects of ionizing irradiation on a cytoplasmic organelle. In parallel with the quantitative investigation, it was found that the resolution of the electron microscope used as a simple morphological tool is of little help in detecting early quantitative changes produced by 1000 r or less. Nevertheless, in animals irradiated with 3000 to 16,000 r, qualitative morphological alterations become quite prominent (Hendee and Alders, 1968).

Several conclusions can be drawn from the observations made in this study. The first is that rat liver mitochondria have a remarkable capacity for rapid increase in total dry mass. The early rise in mitochondrial dry mass is dramatic, and proceeds at a rate that would double mitochondrial mass in less than 12 hours; however this rate levels off at about 3 hours postirradiation. No simple mechanism, such as prevention of mitochondrial division through interruption of the continuous process of DNA synthesis (S. Nass, 1967), could account for this rapid rise in dry mass, because the half-life of a rat liver mitochondrion is as long as about 9 days (Droz and Bergeron, 1968; Beattie *et al.*, 1967; Fletcher and Sanadi, 1961). The conclusion therefore seems justified that X-irradiation has an initial growth-stimulating effect on mitochondria. That this stimulation appears to be limited to the chondriome strengthens the concept that mitochondrial growth is relatively independent of nuclear growth, an autonomy demonstrated for the early synthesis of mitochondrial DNA in *Zea mays* by Van de Walle and Bernier (1967), as well as by the labeling experiments of Reich and Luck (1966).

According to Evans and Norman (1968; also see Spiegler and Norman, 1969), there is a stimulation of thymidine uptake into lymphocytic *nuclear* DNA starting immediately after irradiation with 1 kilorad of X-rays or with ultraviolet light; this effect is of particular note because it

lasts for 6 hours, i.e., about the same length of time observed in our mitochondrial experiments. However, quite opposite findings have been reported by Looney and Chang (1969), after irradiating tumors both in unoperated and in partially hepatectomized rats; a maximum depression of nuclear DNA synthesis was seen autoradiographically 8 hours post-irradiation, and recovery was complete at 24 hours. Interpretation is further complicated by Little's (1968) observation that there is a significant delay in the onset of DNA synthesis in primary human amnion cells after an irradiation dose of 1000 r. None of these studies provides any explanation for the rapid increase of mitochondrial mass or its transiency as observed in this study. Only biochemical investigations can determine whether we are dealing with an unscheduled, and possibly nonfunctional, uptake of organic substances from the cytoplasm followed by their gradual release. However, the proportionality of the "growth" process to the original mitochondrial mass implies the operation of some control mechanism governing an accelerated assembly of preexisting or hastily synthesized components of functional mitochondria and which, because of rate limitations in the extramitochondrial synthesis of two thirds of the mitochondrial components, cannot be sustained and therefore levels off a few hours after irradiation. Only resumption of mitochondrial division would bring the population back to almost normal. These interpretations remain tenuous at best.

As a second conclusion, we regard the increase of mitochondrial dry mass postirradiation as, in fact, "growth." Certainly there are no morphological indicators to suggest that the forms of liver mitochondria which result have anything but classic mitochondrial morphologies. It must be left to biochemical analysis to confirm this concept of growth, because, as this study has shown, visual inspection cannot reveal quantitative mass and volume changes of the order of 20 to 30%.

As a third conclusion, we believe that the evidence from dry mass determinations with the electron microscope, as well as from measurements of nitrogen content per unit volume of mitochondria, show that mass increase involves incorporation or accumulation of organic material within the organelle, and therefore is not to be confused with swelling. A fourth conclusion is that the effect is rapid but *transient*, reaching a maximum at 6 hours and returning to nearly normal values at about 15 to 24 hours; by that time, it can be assumed, systemic effects of the irradiation on bone marrow and gut may begin to reflect secondarily on liver function. Relative to this point, our shielding experiments strongly suggest a fifth conclusion, i.e., that *the effect is direct* and caused by ionizations in the liver itself, or more specifically, in the liver mitochondria.

As a sixth conclusive point, we note that the size distribution of the mitochondrial population is little affected up to 6 hours postirradiation if one regards the lighter subpopulation as a more severely damaged fraction from which material has been lost in the isolation procedure. For the major population of mitochondria with normal densities but increasing dry mass, this means that *the magnitude of the irradiation effect is proportional to the dry mass* that each mitochondrion had at the time of the irradiation insult; the effect is intrinsically proportional to the *size* of the target particle. By contrast, recovery of the increased dry mass to almost normal values does not proceed in quite the same orderly fashion as the initial increase; a wider spread of values was found in the populations studied (Fig. 9).

Since dry mass alone seemed improbable as irradiation target, we investigated the quantitative relationship of mitochondrial dry mass to M-DNA content (Bahr, 1969). Although much information has been published about the concentration of M-DNA relative to mitochondrial protein (cf. comprehensive review by M. M. K. Nass, 1969), only our quantitative electron microscope determinations of dry mass in single mitochondria could reveal that this variable is closely correlated with DNA content per individual mitochondrion (Fig. 10).

Several recent reviews deal with the nature and configuration of mitochondrial DNA (Borst and Ruttenberg, 1966; M. M. K. Nass, 1969; S. Nass, 1969; Schatz, 1970); these summarize the substantial evidence that most, if not all, mammalian M-DNA occurs as circular molecules about 5.3 μ in length. Since one micron of double helix weighs 3.26×10^{-18} gm (cf. DuPraw and Bahr, 1969), the dry mass of such an M-DNA ring can be calculated as 17.4×10^{-18} gm; in Fig. 10, this DNA mass corresponds to a mitochondrial dry mass of 2×10^{-14} gm, a value in good agreement with the mass of the smallest mitochondria found by Glas and Bahr (1966). Extrapolation of this proportionality indicates that the largest mitochondria may contain as many as 70 or more circular molecules.

We propose that one circular M-DNA molecule and the associated dry mass of 2×10^{-14} gm define a unit mitochondrion of rat liver, representing a building block for heavier mitochondria; multiples of this unit would constitute the target volumes in the irradiation experiments described in this article. The unit mitochondrion concept contradicts the idea that the number of genetically active copies of M-DNA is at any time smaller than the number of mitochondria, as suggested by the experiments of Maroudas and Wilkie (1968).

An average rat liver mitochondrion weighs 13.6×10^{-14} gm (Glas and Bahr, 1966). Since there is 0.9 μg of M-DNA/mg of dry mass in

the mitochondria of the livers from our strain of rats, this equals 0.90×10^{-17} gm of M-DNA/10^{-14} gm of dry mass, or 12.2×10^{-17} gm of M-DNA per average mitochondrion. The calculated dry mass of one circular M-DNA molecule is 17.4×10^{-18} gm, and simple division indicates an average of 7 circular M-DNA molecules per average mitochondrion. Earlier Nass (1966) suggested from bulk values that there should be 5 to 6 circular DNA molecules per mitochondrion, with a mean M-DNA content of 8.8×10^{-17} gm.

Alternative calculations should be considered. For example, a somewhat lower mass per micron of DNA (3.19×10^{-18} gm) corresponds to the value of 1.92×10^{6} daltons/μ used by MacHattie and Thomas (1964; see also MacHattie *et al.*, 1965). On the other hand, Suyama and Miura (1968) found 3.7×10^{10} μ of DNA weighing 2.4×10^{8} daltons for the M-DNA of *Tetrahymena* in B-configuration; this equals 1.542 μg of M-DNA $\times 10^{-24}$ per dalton or close to 2.96×10^{-18} gm/μ of the double-stranded molecule. On the basis of these lower masses per unit length for M-DNA, we calculate close to 8 (7.7) molecules for an average rat liver mitochondrion. By contrast, if we use a lower estimate for the M-DNA/protein ratio as determined by other investigators (see the tabulations in M. M. K. Nass, 1969), an average of 4 molecules M-DNA per average mitochondrion can be calculated.

For use in the following calculations, we have chosen a value between 7 and 4, such as 5 molecules per average mitochondrion, and used published figures of 50–60 $\times 10^{10}$ for the total number of mitochondria per gram of wet tissue from rat liver (Baudhuin and Berthet, 1967; Gregson and Williams, 1969); the total M-DNA content of the 7-gm livers used in our experiments would then be 19.25×10^{12} molecules of M-DNA, or about 3.35×10^{-4} gm of M-DNA per liver. A handbook gives a value of about 100 mg % DNA for fresh rat liver (75 to 115). Estimates of the fraction of total DNA represented by M-DNA vary from 0.15% (M. M. K. Nass, 1969) to 1% (Borst, 1967). For a liver weighing 7 gm, the 1% estimate would give a total of 1.43×10^{-4} gm of M-DNA, while the 0.15% estimate would give 0.22×10^{-4} gm. These values are between one fifteenth and one half of the 3.35×10^{-14} gm calculated from the entirely different data of this study.

According to a graph by Corry and Cole (1968), in which radiation dose is related to double-strand breaks per gram of Chinese hamster chromosomal DNA, a value of about 1×10^{14} breaks per gram corresponds to a dose of 1 kilorad; this is in good agreement with the data of Lett and Alexander (1961). Based on these figures, it can be estimated that the number of double-strand breaks should be about 1 in 600 circular DNA molecules (17.4×10^{-18} gm $\times 1 \times 10^{14}$ breaks

per gram). Corry and Cole, as well as Veatch and Okada (1969), estimate the frequency of single-strand breaks to be about an order of magnitude higher, which means a probability that only 1 in 60 M-DNA molecules will undergo such an event. These calculations may be misleading, since they are based on the radiosensitivity of mammalian chromatin; Little (1968) has shown that bacterial DNA is vastly more sensitive than human DNA (see also Freifelder, 1968). The nucleoproteins of mammalian cells appear to possess special radiation protection as compared with the "naked" DNA of bacteria (and perhaps mitochondria). Finally, our seventh conclusion is that both single- and double-strand breaks in M-DNA with subsequent repair replication (Painter and Cleaver, 1967) can account for the observed dry mass effects (provided that the premises for the above calculations are correct).

With this conclusion, the concept that M-DNA is involved attains meaning because of the apparent correlation of the irradiation effects with mitochondrial dry mass, together with the proportionality of dry mass to DNA content. In extensive investigations, Sparrow *et al.* (1967) demonstrated a close correlation between radiosensitivity and nuclear volume or DNA content. Assuming again the extrapolated frequency of 10^{14} double breaks per gram for 1 kilorad, we have calculated that the 1 to 10 million times larger haploid human genome can be expected to suffer roughly 300 double-strand breaks and 3000 single-strand breaks (3×10^{-12} gm of DNA per haploid nucleus $\times$ 10^{14} double breaks per gram of DNA). A specific depression in the uptake of label into M-DNA after 800 r total-body irradiation has been reported by Tamvakopoulos and Van Lancker (1969); however, this effect does not reach a maximum until 18 hours postirradiation, while the dry mass increase in our series peaks at 6 hours postirradiation. Chang and Looney (1966) also reported depression of thymidine uptake after irradiating partially hepatectomized rats. At present the possibility cannot be excluded that any relation between the very early effects of radiation and the DNA content of the organelles may be entirely fortuitous and that ionization of parts of the mitochondrial membrane may actually elicit the early reaction.

Our data, which imply as many as 60 to 70 circular M-DNA molecules in one, large mitochondrion, support the suggestions of other investigators that all the genetic information is contained in one circular molecule, and that multiple M-DNA rings imply genetic redundancy (M. M. K. Nass, 1969; Maroudas and Wilkie, 1968; Hollenberg *et al.*, 1969; Borst *et al.*, 1967). It has also been stated repeatedly (Edelman *et al.*, 1966; Work, 1967) that the capacity for genetic information in one circular molecule of M-DNA is rather limited, and that M-DNA may code

mainly for mitochondrial ribosomes and for the 35 to 45% (Criddle and Schatz, 1969) of structural proteins in the mitochondrion (Pihl and Bahr, 1970; Work, 1967; Bronsert and Neupert, 1967; Roodyn and Wilkie, 1968; Work *et al.*, 1968).

With respect to the unit mitochondrion hypothesis proposed in this article, the circular dimers of 10 μ contour length found by Clayton *et al.* (1969) in neoplastic tissue suggest the possibility that there are normally *two* rather similar mitochondrial chromosomes, which in the malignant state tend to link up as a kind of primitive chromosomal aberration. In a very detailed study by Berger (1968), counts of M-DNA rings in sea urchin eggs were correlated with counts of mitochondria in thin sections; he concluded that there is one circular DNA molecule per mitochondrion and that the percentage of catenated molecules corresponds well with the number of double-size mitochondria. Berger's data support the concept that only one circular molecule constitutes the genome of the mitochondrion.

We have found a ratio of 0.9 μg M-DNA/mg of dry mass in liver mitochondria from the strain of rats used in this study. Different ratios may prevail in different tissues of the same rat, in other species, and in genetic variants of the same species. Variability within a species can be quite pronounced, and the tabulations by M. M. K. Nass (1969) provide an excellent summary of recent data. The ratio is known to change rapidly as much as 130% during chronic administration of testosterone or triiodothyronine, generally by a change in the protein component (De Leo *et al.*, 1969). The M-DNA/protein ratio is also significantly elevated in mitochondria from tumor tissues as compared with normal host cells (Wunderlich *et al.*, 1966; Graffi, 1968).

V. Summary

X-Irradiation of rat livers *in vivo* with 1000 r produces a rapid but transient increase of 30 to 40% in median mitochondrial dry mass after 6 hours. The effect is direct; if the liver is shielded while the rest of the body is irradiated, this dry mass increase is prevented. No single ultrastructural change is specific for the mitochondrial response to irradiation. However, the dry mass increase in any given mitochondrion seems to be proportional to the dry mass present at the time of the irradiation insult. In addition, we have found close proportionality of mitochondrial dry mass to M-DNA content. As a result, this article proposes a *unit mitochondrion* having a dry mass of about 2×10^{-14} gm and containing one circular DNA molecule of 5.3 μ contour length

as the building block for rat liver mitochondria; the largest mitochondria are thought to contain up to 70 *unit mitochondria.*

NOTE ADDED IN PROOF

T. B. Kazakova, M. M. Gachava, and K. A. Markosyan recently published an article [*Biochemistry (USSR)* **34**: 809 (1969)] on thymidine-^{14}C labeled rat liver mitochondria fractionated by differential centrifugation. It was found that radioactive label was highest in the heaviest fraction and decreased in subsequent fractions. The authors assume genetic heterogeneity. Their results are in good agreement with the unit mitochondrion model.

REFERENCES

Baetcke, K. P., Sparrow, A. H., Nauman, C. H., and Schwemmer, S. S. (1967). *Proc. Nat. Acad. Sci. U.S.* **58**: 533.

Bahr, G. F. (1957). Thesis, Karolinska Institutet, Stockholm, Sweden.

Bahr, G. F. (1969). *J. Cell Biol.* **48**: 9a.

Bahr, G. F., and Glas, U. (1964). *J. Cell Biol.* **23**: 8A.

Bahr, G. F., and Zeitler, E. (1965a). *In* "Symposium on Quantitative Electron Microscopy" (G. F. Bahr and E. Zeitler, eds.), pp. 217–239. Williams & Wilkins, Baltimore, Maryland.

Bahr, G. F., and Zeitler, E. (1965b). *Lab. Invest.* **14**: 880.

Bahr, G. F., and Zeitler, E., eds. (1965c). "Quantitative Electron Microscopy." Williams & Wilkins, Baltimore, Maryland.

Bahr, G. F., Carlsson, L., and Lomakka, G. (1956). *Rev. Sci. Instr.* **27**: 749.

Bahr, G. F., Carlsson, L., and Zeitler, E. (1961). *Int. Biophys. Congr., Stockholm,* p. 327.

Baudhuin, P., and Berthet, J. (1967). *J. Cell Biol.* **35**: 631.

Beattie, D. S., Basford, R. E., and Korik, S. B. (1967). *Biochemistry* **6**: 3099.

Berger, E. R. (1968). *J. Cell Biol.* **39**: 12a (Part 2).

Borst, P. (1967). *Biochem. J.* **105**: 37P.

Borst, P., and Ruttenberg, G. J. C. M. (1966). *In* "Regulation of Metabolic Processes in Mitochondria" (J. M. Tager *et al.*, eds.), Elsevier, Amsterdam.

Borst, P., Ruttenberg, G. J. C. M., and Kroon, A. M. (1967). *Biochim. Biophys. Acta* **149**: 140.

Bronsert, V., and Neupert, W. (1967). *In* "Regulation of Metabolic Processes in Mitochondria" (J. M. Tager *et al.*, eds.). Elsevier, Amsterdam.

Burton, K. (1956). *Biochem. J.* **62**: 315.

Chang, L. O., and Looney, W. B. (1966). *Int. J. Radiat. Biol.* **12**: 187.

Clayton, D., Smith, C. A., and Vinograd, J. (1969). *Fed. Proc.* **28**: 532.

Corry, P. M., and Cole, A. (1968). *Radiat. Res.* **36**: 528.

Criddle, R. S., and Schatz, G. (1969). *Biochemistry* **8**: 322.

De Leo, T., Barletta, A., and Di Meo, S. (1969). *Life Sci.* **8**: 747.

Droz, B., and Bergeron, M. (1968). *Proc. 3rd Intern. Congr. Histochem. Cytochem., New York,* p. 42. Springer Verlag, New York.

DuPraw, E. J., and Bahr, G. F. (1969). *Acta Cytol.* **13**: 188.

Edelman, M., Epstein, H. T., and Schiff, J. A. (1966). *J. Mol. Biol.* **17**: 463.

Evans, R. G., and Norman, A. (1968). *Radiat. Res.* **36**: 287.

Fletcher, M. J., and Sanadi, D. R. (1961). *Biochim. Biophys. Acta* **51**: 356.

Freifelder, D. (1968). *J. Mol. Biol.* **35**: 303.

Glas, U., and Bahr, G. F. (1966). *J. Cell Biol.* **29**: 507.
Graffi, A. (1968). *Zentrabl. Gynaekol.* **90**: 945.
Gregson, N. A., and Williams, P. L. (1969). *J. Neurochem.* **16**: 617.
Hendee, W. R., and Alders, B. S. (1968). *Lab Invest.* **18**: 151.
Hollenberg, C. P., Borst, P., Thuring, R. W. J., and Van Bruggen, E. F. J. (1969). *Biochim. Biophys. Acta* **186**: 417.
Kaplan, H. S., and Moses, L. E. (1964). *Science* **145**: 21.
Lett, J. T., and Alexander, P. (1961). *Radiat. Res.* **15**: 159.
Little, J. B. (1968). *Int. J. Radiat. Biol.* **13**: 590.
Looney, W. B., and Chang, L. O. (1969). *Cancer Res.* **29**: 1156.
Loud, A. V. (1962). *J. Cell Biol.* **15**: 481.
Loud, A. V., Barany, W. C., and Pack, B. A. (1965). *In* "Quantitative Electron Microscopy" (G. F. Bahr and E. Zeitler, eds.), p. 258. Williams & Wilkins, Baltimore, Maryland.
MacHattie, L. A., and Thomas, C. A., Jr. (1964). *Science* **144**: 1142.
MacHattie, L. A., Berns, K. I., and Thomas, C. A., Jr. (1965). *J. Mol. Biol.* **11**: 648.
Maroudas, N. G., and Wilkie, D. (1968). *Biochim. Biophys. Acta* **166**: 681.
Nass, M. M. K. (1966). *Proc. Nat. Acad. Sci. U.S.* **56**: 1215.
Nass, M. M. K. (1969). *Science* **165**: 25.
Nass, S. (1967). *Biochim. Biophys. Acta* **145**: 60.
Nass, S. (1969). *Int. Rev. Cytol.* **25**: 55.
Noyes, P. P., and Smith, R. E. (1959). *Exp. Cell Res.* **16**: 15.
Painter, R. B., and Cleaver, J. E. (1967). *Nature* **216**: 369.
Pihl, E., and Bahr, G. F. (1970). *Exp. Cell Res.* **59**: 379.
Reich, E., and Luck, D. J. L. (1966). *Proc. Nat. Acad. Sci. U.S.* **55**: 1600.
Roodyn, D. B., and Wilkie, D. (1968). "The Biogenesis of Mitochondria." Methuen, London.
Schatz, G. (1970). *In* "Membranes of Mitochondria and Chloroplasts" (E. Racker, ed.). Van Nostrand, New York.
Smith, R. E. (1956). *N.Y. Acad. Sci.* **62**: 403.
Sparrow, A. H., and Miksche, J. P. (1961). *Science* **134**: 282.
Sparrow, A. H., Underbrink, A. G., and Sparrow, R. C. (1967). *Radiat. Res.* **32**: 915.
Spiegler, P., and Norman, A. (1969). *Radiat. Res.* **39**: 400.
Suyama, Y., and Miura, K. (1968). *Proc. Nat. Acad. Sci. U.S.* **60**: 235.
Tamvakopoulos, E., and Van Lancker, J. L. (1969). *Am. J. Pathol.* **55**: 1a.
Van de Walle, C., and Bernier, G. (1967). *C. R. Acad. Sci.* **265**: 1599.
Veatch, W., and Okada, S. (1969). *Biophys. J.* **9**: 330.
Weibel, E. R., Kistler, G. S., and Scherer, W. F. (1966). *J. Cell Biol.* **30**: 23.
Work, T. S. (1967). *Biochem. J.* **105**: 38P.
Work, T. S., Coote, J. L., and Ashwell, M. (1968). *Fed. Proc.* **27**: 1174.
Wunderlich, V., Schütt, M., and Graffi, A. (1966). *Acta Biol. Med. Ger.* **17**: 127.
Zeitler, E., and Bahr, G. F. (1957). *Exp. Cell Res.* **12**: 44.
Zeitler, E., and Bahr, G. F. (1962). *J. Appl. Phys.* **33**: 847.
Zeitler, E., and Bahr, G. F. (1965). *In* "Quantitative Electron Microscopy" (G. F. Bahr and E. Zeitler, eds.), p. 208. Williams & Wilkins, Baltimore, Maryland.

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