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VIRAL REPLICATION AND CANCER

Edited by
J. L. Melnick, S. Ochoa, and J. Oró

Proceedings of the
2nd DURAN-REYNALS
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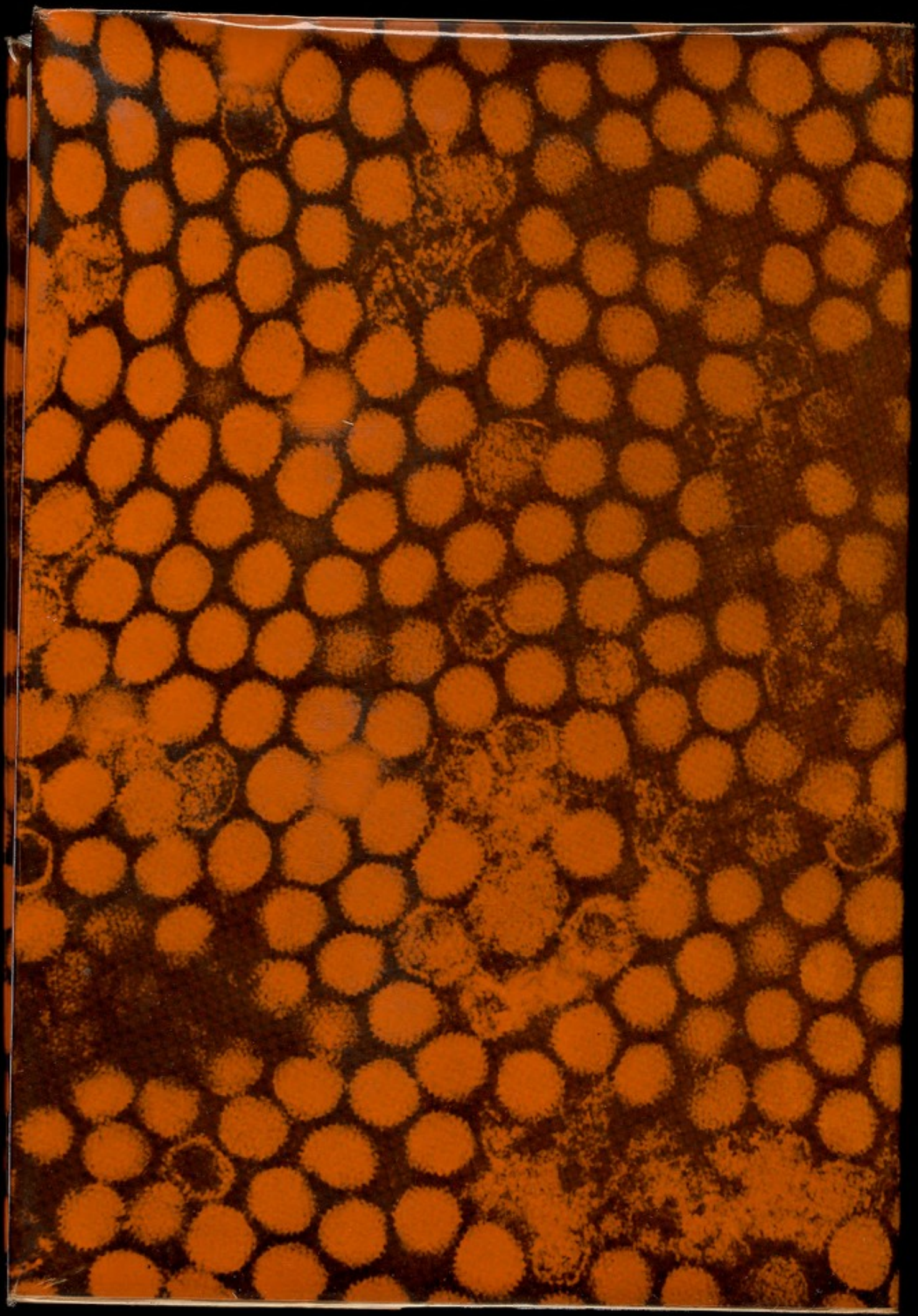

Electron micrograph, kindly supplied by Professor Joseph L. Melnick, of a human adenovirus which contains a DNA genome. The virus is 65 nanometers in diameter and contains 252 capsomers. The virus possesses icosahedral symmetry. The outline of the virus often appears hexagonal; and triangular phases each containing 21 capsomers may be seen. Over thirty antigenic types are known and many have proven to be oncogenic in hamsters.

2nd Duràn - Reynals International Symposium ON VIRAL REPLICATION AND CANCER

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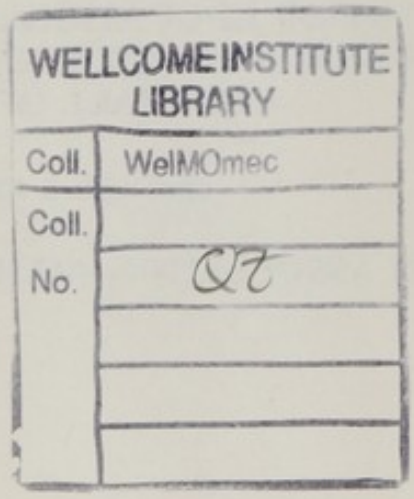

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VIRAL REPLICATION AND CANCER

Proceedings of the 2nd Durán-Reynals Symposium,
June 21-23, 1973, Barcelona, Spain

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FRANCISCO DURÁN-REYNALS, 1945,
at Yale University School of Medicine
New Haven, Connecticut, U.S.A.
(Photograph by J. L. Melnick)

Dedicated to the memory of
Dr. FRANCISCO DURÁN-REYNALS
(1899-1958)

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PREFACE

Because of their relative simplicity the chemistry, biology and genetics of viruses have been extensively investigated and this study has already given us deep insight into molecular events which are the basis of life. This includes understanding of the nature of the genetic material and the mechanisms involved in its replication and in the expression of its informational content.

The growth and multiplication of cells is normally under strict control. We still know little about the nature of the control mechanisms. Cell division occurs after the cell genome, i.e. the DNA, has replicated. When normal cells are cultured on an agar nutrient medium they stop dividing as soon as they form a cell monolayer which occupies the entire surface. Cancer cells, on the other hand, do not stop at the monolayer stage but go on multiplying to form several cell layers. Clearly, the control of growth fails in cancer and the cells multiply without restraint. Control of cell division is likely to be exerted by regulating the replication of DNA and the basic cause of cancer may be a failure of mechanisms of regulation of DNA synthesis.

A number of viruses can convert normal cells into cancer cells, at least in experimental animals or cells in culture, including cultured human cells, and there is compelling evidence for the view that integration of viral genetic material into the cell genome is the primary event in this transformation. This is true whether the transforming virus is a DNA virus or an RNA one. In the latter case, the presence in the virion of reverse transcriptase, an enzyme catalyzing synthesis of DNA on an RNA template, gives rise to virus-specific DNA whose incorporation into the cell's DNA may bring about tumoral transformation.

To the theoretical interest in the study of the molecular biology of viruses the hope is now added that this study, particcularly in the case of oncogenic viruses, may lead to an understanding of the fundamental altera-

tion(s) responsible for the unrestrained growth characteristic of cancer. Hopefully this knowledge may help us find means to effectively combat this disease; it can also give insight into as yet unknown basic molecular aspects of the biology of the cell.

It is difficult to predict which particular line of investigation will lead to a breakthrough in knowledge on the basic nature of cancer but, since it is conceivable that such a breakthrough may require new knowledge of normal cell functions we stand a better chance of arriving sooner at a knowledge of the basic molecular alteration or alterations in cancer by conducting the attack over a broad front. Thus, areas of endeavor aimed at increasing knowledge of the molecular mechanisms of replication of bacteriophages, the mechanisms controlling or regulating this replication, and the primary sequence and secondary structure of viral RNA, which are vital for a real understanding of translational controls, may be of paramount importance in the overall effort to understand the nature of malignant transformation. In line with the above philosophy this symposium covers a wide and exciting front of research on the nature of the viral genome, the mechanisms of viral replication, and viral oncogenesis.

Contained in this book are the papers presented at the 2nd Francisco Durán-Reynals Symposium in his native city of Barcelona, June 21-23, 1973. It is fitting that the Symposium is entitled "Viral Replication and Cancer", since for many years he was the leading and almost the sole exponent of the view that viruses play an important role in causing cancer in many species, including humans. It is a particular pleasure to have as one of the contributors of the Symposium María Luisa Durán-Reynals who worked with her husband and who has continued to move his experiments forward in the area of viral oncogenesis. All of the participants are leaders in the field of virology.

The first session of the Symposium is concerned with fundamental molecular aspects of viral replication. Indeed, the structure and function of viral genomes themselves are now amenable to attack in the laboratory. High-voltage, low-temperature electron microscopy is adding to the insights on virus structure and advancing the possibility of correlating viral structure and function.

The second session focuses on molecular biology of animal viruses, on the use of temperature-sensitive mutants for analyzing viral genetic functions, and particularly on the in vitro *synthesis of cellular genes by the use of reverse transcriptase obtained from oncornaviruses.*

The third session broadens the discussion of viral oncogenesis and is concerned with the interaction of chemical carcinogens and viruses in

causing cancer, with the state of the viral genome in cancer cells, with the inheritance of viral genes and with the possibility that viruses widespread in the population —such as herpesviruses— may play a causative role in cancer by themselves or in consort with other factors.

At this time we wish to remember with respect and affection Wendell M. Stanley, a pioneer of virus research and a firm believer in and persuasive promoter of the hypothesis of the viral etiology of cancer. Stanley was Co-Chairman, with J. Casals, of the 1st Durán-Reynals Symposium, held in Barcelona in June 1971, to which he contributed the first paper, a masterful introduction to the past and present of the relation between viruses and cancer. With his passing away in Salamanca shortly before he was due to return to the U.S.A., we lost not only a staunch supporter of the study of viral oncology but also an ardent defender of the cause of science for its own sake.

We wish to express our appreciation to the Instituto de Biología Fundamental of the Universidad Autónoma de Barcelona and the Department of Biophysical Sciences of the University of Houston for organizing this meeting and bringing the participants in the Symposium together in the historic city of Barcelona, where Francisco Durán-Reynals was born and started his intellectual life and scientific work.

The Symposium has been held under the auspices of the Ministerio de Educación y Ciencia, Consejo Superior de Investigaciones Científicas and the Excmo. Ayuntamiento de Barcelona, with the collaboratorion of the Sociedad Española de Bioquímica and the Acadèmia de Ciències Mèdiques de Catalunya i Balears and with a special grant from the Junta Provincial en Barcelona de la Asociación Española contra el Cáncer.

It is also a great pleasure to acknowledge the enthusiastic collaboration of Luís Cornudella, Julio R. Villanueva, Daniel Navarro as well as Verle Rennick and Marianna O'Rourke in the organization of the Symposium, editing the manuscripts and reading proof. Our thanks are due to the staff of Editorial Labor for their careful printing of the book.

JOSEPH L. MELNICK
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Introduction to the 2nd Duran-Reynals International Symposium on viral replication and cancer

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During this century we have witnessed the rise and fall of various exclusive theories of carcinogenesis, including the acceptance of oncogenic viruses as the sole cause of certain cancers. The increasing realization of the molecular and genetic complexity of the vertebrate cell has indeed hindered those seeking to identify the molecular mechanism by which such cells are rendered malignant. However, the advances made in recent years in defining the genetic composition of oncogenic viruses and in elucidating the interactions between viral and cell genes are beginning to provide significant insights into the problem of carcinogenesis at the cellular level. Indeed, one of the most exciting recent developments has been the recognition of heritable genes of the oncogenic RNA viruses, or oncornaviruses, in normal vertebrate cells and their activation by chemical carcinogens. FRANCISCO DURÁN-REYNALS, the man we are assembled to honor here, would have been most gratified to have been able to witness these developments, for in large measure, they reflect his incisive and visionary work of many years ago devoted to the interplay between viruses and chemical carcinogens.

In the papers presented in this Symposium, the current state of the art in studies of viral replication and cancer is reported by investigators who are working at the growing edge of viral oncology; much of this work stems directly or indirectly from the pioneering work of FRANCISCO DURÁN-REYNALS. Francisco would not be surprised at these new developments – but he would be fascinated and exhilarated!

It was a great personal pleasure to have been associated with DURÁN-REYNALS at Yale and to have come under his influence. The photograph of FRANCISCO DURÁN-REYNALS which appears as the frontispiece of this volume was taken almost 30 years ago when we joined forces to offer a course in virology at Yale University, and serves to remind us once again of the vital, creative and thoroughly human being whom we honor here. I am particularly pleased that the participants in this Symposium include his wife and scientific collaborator, MARÍA LUISA, who has continued to carry their work forward. All of us who were blessed by the friendship of Francisco and María Luisa know that the warmth and sparkle of the DURÁN-REYNALS' home in New Haven reflected them both.

It is significant that a line of investigation begun by FRANCISCO DURÁN-REYNALS more than 20 years ago, and being pursued today by MARÍA LUISA DURÁN-REYNALS, is still very much in the mainstream of virus-cancer research. This team was the first to demonstrate that a cytolytic virus infective to man enhances the neoplastic effect of a chemical carcinogen. In this Symposium, MARÍA LUISA DURÁN-REYNALS reports on the investigations of her own laboratory and of others in efforts to unravel the combined neoplastic effects of vaccinia virus, of 3-methylcholanthrene and of genetic host factors.

The life and scientific career of DURÁN-REYNALS, and some of his most important papers, were set forth in the First International Symposium dedicated to him here in Barcelona. It is fitting that the author of the first presentation in this Second Durán-Reynals International Symposium is a distinguished and internationally recognized scientist who, as a newly graduated doctor arriving at Francisco's Yale laboratories 24 years ago, found not only a wealth of scientific inspiration, but also personal warmth and "sheer friendliness." OKER-BLOM briefly reviews some of the highlights of that career, and describes some of his experiences as a student and young collaborator with DURÁN-REYNALS.

The debt owed by animal virology to bacteriophage investigators is clearly seen in two chapters dealing with phage replication and genetics at the molecular level. WEBER, BILLETER and WEISSMANN discuss the replication of RNA phages, with emphasis upon phage $Q\beta$ and the aspects of its replication having special significance for other viruses as well. Three interrelated features stand out: the economy of genetic information and the evolution of virus-specific components to perform more than one function in replication; the ability of the viral replicative system not only to utilize the synthetic machinery of the cell but also to divert a number of host components to specific viral functions which appear quite unrelated to the cellular function that these components normally fulfil;

and a complex regulatory system, in which both synthesis and function of each viral component is affected by interactions with other components. Even though it is one of the smallest of the viruses, Qβ emerges as a surprisingly sophisticated creature.

The intensive study of RNA bacteriophages in the past decade has provided relatively simple model systems not only for studies of viral RNA replication, but also for approaching fundamental questions such as translation and gene control mechanisms. The small RNA genome of bacteriophage MS2 contains only three genes, coding for the A-protein, the coat protein, and the replicase subunit, but it is far more than a mere linear information tape. The RNA functions in an intricately controlled translation system, in replication, in virus assembly, and in the process of preempting the host cell metabolism. FIERS and his colleagues have made great strides in determining the primary structure of the viral RNA, and many important regions of MS2 RNA have been sequenced. The complete primary structure should be established soon, although FIERS warns that full understanding of the secondary and tertiary structure seems still far in the future.

Just as plant virology already has contributed to fundamental understanding of virus self-assembly, research in the field can be expected soon to make important and unique contributions to basic understanding of virus replication. Plant viruses with divided genomes have multiple RNA components, as do many animal viruses, but in these plant viruses the RNA components are independently encapsulated to give a virus which requires two or three different particles to produce infection. This unique phenomenon is apparently confined to certain groups of plant viruses. Among them the bromoviruses are the best characterized, and LANE relates some of the approaches and accomplishments of the past few years which have greatly changed our view of their biology. The way now appears to be opened, particularly for studies of the potential of plant viruses to show phenotypic variation, and for learning how individual mutations alter the phenotypes.

Electron microscopy has already made possible the direct visualization of structures at the molecular level, and has formed the basis of our understanding of virus structure. However, new approaches in instrumentation and preparation have been needed in order to overcome the limitations imposed by the instrument, particularly to move toward the electron microscopic study of biological systems in their native state. FERNÁNDEZ-MORÁN is among those who have brought us closer to this goal. He reviews recent developments culminating in the first functioning prototype for a large-scale cryo-electron microscopic system in his own

laboratory which permits resolutions of 8 to 16 angstroms under conditions approaching the native hydrated state. Applications of these advanced electron microscopic methods to the study of macromolecular organization of biologic systems are described and well illustrated. The author discusses applications to ultrastructure of viruses, DNA molecules and enzymes, and to hydrated membranes. Electron microscopy is on the verge of correlating structure and function at the molecular level, and we look forward to these new approaches that combine a high degree of resolution and specimen preservation under native conditions.

For almost every group of animal viruses, there have been reports of defective particles which interfere with the replication of homologous non-defective virions. The first separation and positive identification of these defective interfering particles was accomplished with vesicular stomatitis virus, by HUANG and her colleagues. In this symposium, she summarizes data from the VSV system to illustrate the nature and behavior of the defective particles, discussing what is known of their genesis, their physical and biological properties, and the molecular basis of interference. In the short time since their identification, much has been achieved in characterizing these particles and their RNA. The author predicts that further studies on the molecular biology of the defective particle of VSV should contribute to understanding not only of macromolecular synthesis and control, but also of the pathogenesis of viral diseases.

The togaviruses, which include most arboviruses of serological groups A and B, are of special interest to the molecular virologist. Although they manifest a complex biogenesis and morphogenesis, the togaviruses are relatively simple biochemically, their virion being composed of two structural proteins —one in the envelope and one in the nucleocapsid. With most of the antigens contained in the viral envelope, relationships of virus structure and composition to function are accessible to study. After reviewing the structure, composition and replication of the togavirion, GOLDBLUM describes current studies of the immunological functions of subviral components of togaviruses that have been made possible by new methods of dissociating virion components.

The molecular biology of reovirus is more fully understood than that of most other animal viruses. JOKLIK discusses the nature of this extraordinary virus which has a genome of segmented double-stranded RNA, particularly in respect to unusual events which follow penetration of the infected cell, in which the viral genome does not separate from its capsid but in which subviral particles are generated that are capable of transcribing the double-stranded genome into plus-stranded RNA. He describes how the viral protein and RNA are synthesized in infected cells

and go on to form the progeny double-stranded genomes. Many virologists agree that efforts to understand the interaction of tumor viruses with host cells will require information like that which has been developed for conventional infectious viruses and particularly for reovirus.

The recent discovery of the enzyme, reverse transcriptase, which is present in purified oncornavirions and capable of transcribing viral RNA to DNA *in vitro,* strengthened the assumption that the RNA tumor viruses replicate through a DNA intermediate. One of the co-discoverers of this enzyme, BALTIMORE, with his colleagues, VERMA, FAN and TEMPLE, presents recent work with this RNA-instructed DNA polymerase. This unique enzyme utilizes a wide variety of polymers as templates; for the copying of the template to take place, a primer or initiator is required which binds to the template by hydrogen bonds, and is then covalently attached to the newly synthesized DNA. In their recent studies the authors utilize as template the messenger RNA (mRNA) for rabbit globin purified from rabbit reticulocytes and have now successfully achieved synthesis of DNA complementary to the 10S reticulocyte mRNA. The DNA transcript is an accurate copy of the 10S mRNA, because it hybridizes with it. Both single-stranded and double-stranded DNA products have been obtained and both can be transcribed back into mRNA by a conventional RNA polymerase. Thus, we see here, with all its implications for the future, the synthesis of a gene apparently identical to that found within cells.

Temperature-sensitive (ts) mutants have been used to best advantage thus far in the definition of viral gene functions in animal virus systems, as well as for the study of the roles that specific gene products play in viral replication. Extraordinary interest in recent genetic studies has been focused on herpesviruses, since some have been associated with malignancies in man and animals. As discussed by BENYESH-MELNICK, SCHAFFER, COURTNEY and ARON, isolation and characterization of ts mutants of herpes simplex virus is leading not only to the definition of the genes coding for structural viral components, but also to the identification of virus-coded enzymes with viral replicative functions. Furthermore, if only a part of the viral genome is expressed in herpesvirus-transformed cells, as has been shown to be the case with other DNA tumor viruses, the availability of well-characterized ts mutants of herpesvirus offers the possibility, by complementation analysis, of identifying the viral gene functions required for neoplastic transformation.

It is encouraging to have a Nobel Laureate turn his attention to the problems of cancer etiology. CALVIN points out that when he entered this field, with his chief concern in areas of chemical carcinogenesis, he found

inspiration and early evidence for concepts of synergism between chemical and viral carcinogenesis in the work of DURÁN-REYNALS. His recent experiments have been directed toward understanding the mechanism of carcinogenesis and toward a rational approach to cancer chemotherapy by the use of specific enzyme inhibitors.

Among tumor cells resulting from transformation by DNA-containing oncogenic viruses, probably the best-characterized are transformed cells which have been induced by the papovaviruses, SV40 and polyoma virus, but which no longer produce infectious virus. In the report by MELNICK & BUTEL, the experimental observations which led to current concepts regarding the state of the incompletely expressed viral genome within the SV40-transformed cell are considered, and directions of present research in this area are discussed. The evidence is strong that in SV40-transformed cells the viral genome is integrated into the chromosomal DNA of the host cell, and it appears that in most transformed cell lines, the complete viral genome is present; the number of copies of that genome present per cell is, however, still the subject of some controversy. Among currently unresolved areas related to SV40-transformed cells is the mechanism by which the integration of the viral genome is maintained. Approaches being taken toward clarification of this question include the use of defective hybrid viruses, temperature-sensitive virus mutants, and bacterial restriction enzymes. In addition to providing insight into the process of virus-induced transformation, resolution of this problem should also extend our understanding of regulatory mechanisms in the normal mammalian cell.

Implicit in FRANCISCO DURÁN-REYNALS' work and ideas on chemical and viral carcinogenesis was the assumption that tumor virus genes were present in apparently uninfected cells. Recent studies on latent oncornavirus genomes have led to new speculation on their role in cancer, in the form of the oncogene hypothesis and the protovirus hypothesis. In his chapter, WEISS reviews the evidence for inherited viral genomes in mice and chickens and describes the present state of information about how the genomes are controlled by endogenous and exogenous factors. Of interest is his recent finding that genetic recombination can occur between non-defective strains of Rous sarcoma virus and the endogenous viral genome of the cell. Recombinants were obtained that carry the Rous virus transforming genes and the host range gene of the endogenous virus. In addition, normal murine and avian cells can regularly be induced to release complete oncornavirus or virus-specific products (gs antigen) after treatment with chemical or physical carcinogens and mutagens.

A number of herpesviruses now have been associated with neoplasia,
 but the strength of their candidature as etiologic agents varies. It has been

recognized for many years that herpesviruses, in addition to being able to establish a productive, lytic infection in cells, may also establish latent infections, in which the genome may remain intact without producing progeny for long periods, particularly within a non-replicating cell such as the neurone. However, for all or part of a virus genome to be present in every cell of a rapidly multiplying tumor, it must multiply in harmony with the cell, presumably by integration into host cell chromosomes. WILDY surveys the evidence that has been developed and the problems that are encountered in attempts to establish an oncogenic role for a number of herpesviruses: Lucke's virus of frogs, Marek's disease virus of chickens, herpesviruses of rodents and herpesviruses of subhuman and human primates; the latter include EB virus associated with lymphoma and herpesvirus type 2 associated with cervical carcinoma. He concludes that three —Marek's disease and two monkey herpesviruses, can be said to have a causative relationship to neoplasia, while for EB virus and Lucke's virus, the causative relationship is probable but not yet formally proven —perhaps immunological or other cofactors are involved. Work on cervical carcinoma is intensifying and hopefully as the disciplines of molecular virology, immunology and epidemiology are focused on the problem, an answer will be forthcoming soon on whether genital herpesvirus plays a causative role in cervical cancer.

FRANCISCO DURÁN-REYNALS

In Memoriam

NILS OKER-BLOM

Department of Virology, University of Helsinki, Helsinki, Finland

"¿Qué es la vida? un frenesí;
¿Qué es la vida? una ilusión,
una sombra, una ficción,
y el mayor bien es pequeño;
que toda la vida es sueño,
y los sueños, sueños son."

CALDERÓN DE LA BARCA

For one who had the privilege of working as a student and collaborator in his laboratory, remembering FRANCISCO DURÁN-REYNALS and rereading his papers and correspondence is both a great experience and a privilege. FRANCISCO DURÁN-REYNALS must have had some of the scientific vision that is comparable with the talent expressed in the poetry of his great compatriot CALDERÓN DE LA BARCA.

FRANCISCO DURÁN-REYNALS was born in Barcelona on the 5th of December in 1899. His father was the distinguished writer and artist MANUEL DURÁN DURÁN and his mother was Dña. INÉS REYNALS MALLOL. FRANCISCO DURÁN-REYNALS was the youngest of five sons born into this highly talented family. In 1917, he entered the Medical Faculty, from which he graduated in 1925. During the years 1925 to 1926, FRANCISCO DURÁN-REYNALS worked in the Pasteur Institute in Paris in the Department of Professor A. BESREDKA and Professor E. WOLLMAN, and from 1926 at the Rockefeller Institute in New York in the Department of Cancer Research headed by Dr. J. B. MURPHY. In 1938, he moved as a research professor to the Yale University School of Medicine, New Haven, Connecticut, U.S.A., where he stayed until his untimely death in 1958.

The volume dedicated to him and written for the first Durán-Reynals Symposium on Virus and Cancer in Barcelona (June 7, 1971), edited by

W. M. STANLEY, J. CASALS, J. ORÓ and R. SEGURA, presented the life and scientific career of FRANCISCO DURÁN-REYNALS. The significance of his work was also expertly analyzed by his great compatriot, JORDI CASALS, in the same volume, which contains some of DURÁN-REYNALS' most important papers.

All I would wish to do here is offer a collection of my impressions of a happy and scientifically rewarding year in FRANCISCO DURÁN-REYNALS' laboratory at Yale University in 1949-1950. In short, I merely wish to put down some thoughts about his very personal and visionary views.

This newly graduated doctor from remote Finland was deeply impressed with the first meeting he had with FRANCISCO DURÁN-REYNALS in late August 1949, who had just returned from summers spent working and writing as a fellow in the Jackson Memorial Laboratory in Bar Harbor, Maine. His noble looks, his vivacity and his relaxed way – both physically and mentally – of greeting a complete stranger made an indelible impression of a personality exuding intelligence, imagination and, also very important, sheer friendliness. My first impressions were soon confirmed during the year that I spent in FRANCISCO DURÁN-REYNALS' laboratory in New Haven, and they have never changed since.

A particularly memorable event for me was the first postgraduate course in virology given at Yale University with FRANCISCO DURÁN-REYNALS, J. L. MELNICK and B. A. BRIODY as the teachers of a group of six students working in very disparate fields of virology. I would add that the team of FRANCISCO DURÁN-REYNALS included IVAN PARFENTJEV who was working on immunological problems, JEANETTE OPSAHL who was working on the spreading factor, and R. H. PEARCE who assisted with the chemical work, to say nothing of an efficient secretary, several skilful technical assistants and animal keepers who devoted their time and interest to FRANCISCO DURÁN-REYNALS' work. In short, we were, to use banality without being banal, one big family.

This, then, was the "milieu" in which FRANCISCO DURÁN-REYNALS celebrated his half century, guided his students and co-workers, wrote many of his most important papers and reviews. These few rooms became home for us from the outside, not least because MARÍA LUISA, FRANCISCO DURÁN-REYNALS' wife, also worked there. Little wonder that he was totally unsurprised that the wife of this Finnish student should also take up work in the laboratory when she arrived in December, 1949. And outside the laboratory it was the same. MARÍA LUISA and her lovely daughter, FRANCISCA, welcomed us in their home, at many memorable occasions.

FRANCISCO DURÁN-REYNALS' two main areas of investigation were the discovery of and work on the spreading factor and the study and theory of the viral aetiology of cancer. It was in 1928 that FRANCISCO DURÁN-REYNALS presented his first paper on the spreading factor,

"Exaltation de l'activité du virus vaccinal par les extraits de certains organes" (Comptes rendus de la Societe de Biologie de Paris *99:* 6, 1928). This was followed by a number of papers and an extensive review in 1942, "Tissue permeability and the spreading factor in infection" (Bact. Rev. *6:* 197-252, 1942). Later, the phenomenon was the topic of several conferences and symposia, among them the Conference on "The Ground Substance of the Mesenchyme and Hyaluronidase" held at the New York Academy of Sciences in 1948, the papers of which were published in Annals of the New York Academy of Sciences *52,* 1950, and the Symposium on the "Mechanism of Inflammation" held in Montreal in September, 1953. Great interest was focused on the spreading factor for many years thereafter, and FRANCISCO DURÁN-REYNALS obtained well-deserved credit for his discovery. He was asked to write several introductory lectures, reviews and text-book chapters concerning his work and his views on the role of the ground substance as a fundamental factor in natural resistance to infection and cancer, and his name will always be connected with the biochemistry of inflammation.

As early as in the 1930's, he had turned his interest to cancer. The fact that some work on the spreading factor was also going on in the laboratory in 1949-1950 perhaps was due in part to his feeling that some interesting connection between the two problems so close to him might be found. As a matter of fact, he returned to them both in his last papers on the combined effect of carcinogens, hormones and viruses on tumour induction. Most of his work in these years, however, was concerned with the tumour viruses, and in April, 1950 his large review with the provocative title "Neoplastic infection and cancer" appeared (Amer. J. Med. *8:* 490, 1950). This was to be followed by two other reviews: "Virus-induced tumours and the virus theory of cancer" in "The Physiopathology of Cancer", edited by F. HOMBURGER and W. H. FISHMAN, Hoeber-Harper/New York, 1953, and "Realities and hypotheses of viral infection as a cause of cancer" (Revue de Biologie *14:* 411-428, 1956), which is based on the "Louis Berger" lecture delivered at the Tenth Annual Meeting of the Quebec Association of Pathologists in Montreal April 29, 1955. Both the latter review and the revised chapter of the former (in "The Physiopathology of Cancer" edited by G. HOMBURGER, Hoeber-Harper/New York, second edition, 1959) contain the essence of his theory of cancer. The theory was based partly on his own work and partly on ingenious re-interpretation of others' experiments and results.

After working for some time on different aspects of chicken tumours, in 1940 he described the haemorrhagic disease induced in young chicks by sarcoma viruses ("A hemorrhagic disease occurring in chicks inoculated with the Rous and Fuginami viruses", Yale J. Biol. Med. *13:* 77-99, 1940). A similar experiment was immediately done using rabbits and the Shope fibroma virus, which resulted in comparable events ("Production

of degenerative inflammatory or neoplastic effects in the newborn rabbit by the Shope fibroma virus", Yale J. Biol. Med. *13:* 99, 1940). These findings served as the basis for his concept of the dual effect of tumour viruses —that cancer viruses can cause either necrosis or neoplastic lesions depending on the age of the host or on the experimental conditions. Since tumour virus could thus behave more or less like "ordinary" viruses, the opposite was, in his opinion, logical— that is, that so-called "ordinary" viruses could probably, under certain conditions, behave like tumour viruses. This notion was one of the main points of his concept of neoplastic infection.

The other very important point in his discussions was the question of the species specificity and variability of tumour viruses. These problems were tackled in some of his early work on the adaptation of Rous sarcoma virus to other avian hosts and the resulting variation in the virus ("Age susceptibility of ducks to the virus of the Rous sarcoma and variation of the virus in the duck", Science *93:* 501, 1941 and "The reciprocal infection of ducks and chickens with tumor-inducing viruses", Cancer Res. *2:* 343, 1942).

Much of FRANCISCO DURÁN-REYNALS' later work was devoted to the solution of these two problems, and actually our work in 1949 and 1950 was concerned with the haemorrhagic disease induced by variants of the Rous sarcoma virus ("Behaviour of virus from chicken and duck tumours in embryonated eggs of chickens and ducks", Acta path. microbiol. scand. *28:* 1, 1951). A pertinent problem connected with the question of the age of the host was the occurrence of neutralizing antibodies to avian tumours in seemingly healthy birds. FRANCISCO DURÁN-REYNALS pointed out: "These facts, which in avian cancer parallel what is observed in so many infectious diseases, may well be considered an indication of a subclinical infection of the chickens either by the tumour viruses themselves or by other viruses antigenically related to them". The Regional Poultry Research Laboratory at East Lansing, Michigan, had studied chicken lymphomatosis for several years and attempted to obtain lymphomatosis-free chickens by hatching and rearing inbred chickens in family units. FRANCISCO DURÁN-REYNALS immediately recognised the significance of keeping chickens in isolation in order to compare the susceptibility to Rous sarcoma virus of chickens free of lymphomatosis with that of infected chickens. And so, in 1949-1950, the animal rooms were equipped with efficient isolation units. In collaboration with the group in East Lansing, a study was then made on the frequency of antibodies to Rous sarcoma virus and on the suppression of the take of tumours in immunized or naturally immune hosts. ("Studies on the origin of the naturally occurring antibodies against tumor viruses developing in aging chickens", Cancer Res. *13:* 408, 1953). The summary of this paper contains the following statement: "Suggestive, though not entirely

conclusive, evidence of a relationship between lymphomatosis and Rous sarcoma viruses was obtained from experiments designed to test the hypothesis that the two agents possess common antigens". The antigenic relationship between the leukoviruses was definitively established several years later.

One of the concepts adopted by FRANCISCO DURÁN-REYNALS to explain the disappearance of Rous sarcoma virus in the ageing host was the "masking" of the viruses. ANDRÉ LWOFF pointed out in his introductory lecture to the Conference on "RNA Viruses and Host Genome in Oncogenesis" held in Amsterdam in 1971 ("RNA viruses and host genome in oncogenesis", edited by P. EMMELOT and P. BENTVELZEN, North Holland Publishing Company, Amsterdam-London 1971 that FRANCISCO DURÁN-REYNALS was the first to take advantage of the work on bacteriophages. In his review "Virus induced tumors and the virus theory of cancer", he discussed in detail the similarity between lysogeny in bacteria and the masking of tumour viruses and assumed that tumour viruses could occur either in their vegetative form or as some form of provirus. He also drew attention to the fact that prophage could change to virulent phage either spontaneously or following irradiation or treatment with nitrogen mustard, etc.—in other words, treatment resulting in the "unmasking" of virus. This discussion is actually closely connected with the third line of his work, the combined effect of viruses and carcinogens.

By 1938, AHLSTRÖM and ANDREWES had shown that tar or carcinogenic hydrocarbons could activate the Shope rabbit fibroma, resulting in generalized lesions ("Fibroma virus infection of tarred rabbits", J. Path. Bact. *47:* 65, 1938). One of the major postulates of FRANCISCO DURÁN-REYNALS' cancer theory was that the "unmasking" of latent or even ordinary viruses under certain conditions could result in tumour growth, and he had even in his 1950 review suggested that viruses may be "the proximal cause of the tumours resulting from injection of chemicals". Series of experiments to test this started in 1947 and were continued after 1958 by his widow, MARÍA LUISA. The first report published was "Studies on the combined effect of fowl pox virus and methylcholanthrene in chickens" (Ann. N.Y. Acad. Sci. *54:* 977, 1952). The painting of the skin with methylcholanthrene resulted in the activation of latent fowlpox virus in practically every chicken. Continuation of the painting resulted in different tumours, even carcinomas in the treated skin, from which fowlpox virus was often, but not invariably, obtained.

FRANCISCO DURÁN-REYNALS was always very cautious in his conclusions and the discussion ended as follows: "If it appears that fowlpox virus really was instrumental in the development of some or all of the lesions described, the thesis would have been proved that an ordinary virus, activated by a carcinogen, can cause cancer. The field then would be open to investigate other ordinary viruses as possible causes of neoplasia;

and to look for such viruses not as riders but as causative agents, in tumors and other tissues of the cancerous host".

A similar study including treatment with cortisone was performed on mice painted with methylcholanthrene and infected with vaccinia virus. The combined treatment resulted in "much enhanced dermal infection", which was frequently followed by "the development of a variety of benign and malignant neoplastic lesions arising strictly from the precise site (still evident from the scar) injected with the virus material" ("Preliminary studies on the development of neoplasia in the skin of mice painted with methylcholanthrene and injected with cortisone and vaccinia virus", Ann. N.Y. Acad. Sci. *68:* 430, 1957). These findings were later confirmed by MARÍA LUISA DURÁN-REYNALS and clearly showed the increased incidence of tumours in hosts pretreated with vaccinia virus.

In his "Louis Berger" lecture in 1955 FRANCISCO DURÁN-REYNALS summarized his conception of the mechanism of cancer viruses and the attendant infection as follows:

> *"(A)* Selective or exclusive susceptibility of the immature host generally followed by a long period of latency;
> *(B)* Virus variation resulting in adaptation to non-susceptible races, strains or species;
> *(C)* Induction of varied types of malignancy resulting from the above adaptation; and
> *(D)* Induction by some of the viruses of either non-neoplastic or neoplastic lesions, depending on the age of the host or experimental conditions."

Looking at the theory of FRANCISCO DURÁN-REYNALS presented 20 years ago in the light of present knowledge of viruses and tumour viruses in particular, one is struck by his scientific intuition. The number of conferences and papers concerning tumour viruses is now so great that it is difficult for anybody to summarize more than a part of the problem satisfactorily. This alone would, of course, have satisfied FRANCISCO DURÁN-REYNALS, amply justifying his often cited comment: "Viruses are in the cancer problem to stay". He would have had still more satisfaction, however, in the realization that many of the results obtained have either confirmed or added supporting data to his ideas. He would have been interested in the effect of interferon on tumour viruses, since he actually promoted work on these lines in 1950 although for practical reasons it was not completed in his own laboratory ("The effect of Coxsackie virus on the growth of the Rous sarcoma in embryonated chicken eggs", Ann. Med. exp. Biol. Fenn. *34:* 293-308, 1956). The effect of carcinogens on interferon production may have elucidated some of the questions concerning the combined action of carcinogens and viruses.

And he would certainly have taken part with great enthusiasm in the discussions of provirus and protovirus hypotheses and the oncogene theory of cancer and the possibility of rescuing or (as he certainly would have put it) "unmasking" C type particles from several types of tissues by different means.

He would have been particularly satisfied by LWOFF's discussion of the interaction between host and viral genomes which concludes: "Thus, it appears probable that within a few years the existence of a provirus of DNA and RNA oncogenic viruses will be demonstrated. The problem of inhibition and induction of viral function of course remains. This is to my mind one of the key problems of cancer research."

In spite of all efforts and some promising hints, the problem of human cancer is, however, still unsolved. This brings to mind FRANCISCO DURÁN-REYNALS' last paper entitled "The pulmonary adenomatosis complex in sheep" (Ann. N.Y. Acad. Sci. *70:* 726, 1958), and the discussion in that paper on similarities between the "slow virus infections" and cancer. The causative agent of pulmonary adenomatosis in sheep is now believed to be a herpes-like virus; the viruses of Maedi and Visna which contain reverse transcriptase and transform cell cultures are tentatively grouped with the oncornaviruses; the agents of scrapie, Creutzefeld-Jacob disease and Kuru are believed to be either viroids or parts of membranes. Typical for all the viruses or agents, however, is a very long incubation period and some of the last-mentioned viruses need up to 8 years to induce disease in the experimental animals inoculated. The activation of such viruses in ageing has recently been discussed by GAJDUSEK ("Slow virus infection and activation of latent infections in ageing", Advances in Gerontological Research, vol. 4, p. 201, 1972).

FRANCISCO DURÁN-REYNALS was always searching for models which could be used to support his theory. The comparison with the slow virus infections may be far-fetched. These viruses do not comprise a homogeneous group, and even the viruses of pulmonary adenomatosis of sheep and Maedi, which he particularly compared, are apparently different. However, the Maedi virus seems to be very close to the tumour viruses. The comparison may, after all, be another example of FRANCISCO DURÁN-REYNALS' intuition.

FRANCISCO DURÁN-REYNALS' dream was to create a unitary cancer theory. He did more than most of his contemporaries to keep the virus theory alive. Still today, twenty years later, we must acknowledge that this is yet a dream as regards human cancer, although now something more substantial may have been found to bring it to reality.

However this may be, I would use my old teacher's own words in paraphrase: The memory of FRANCISCO DURÁN-REYNALS is permanently imprinted in the world of medical science.

STUDIES ON THE MOLECULAR BASIS OF VIRAL REPLICATION

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Studies on the molecular basis of viral replication

I. Introduction

First of all, I would like to express how much I appreciate the honor of having been invited to contribute to this symposium on viral replication and cancer, held in the memory of the great scientist FRANCISCO DURÁN-REYNALS.

I have been asked to speak about the molecular basis of viral replication. Now it is obviously quite impossible for me to develop here a comprehensive view of this vast field. What I will try to do, however, is to discuss viral replication in *a model case*. The small RNA phages are among the simplest of all viruses, and our knowledge of their replication at the molecular level is probably the most advanced compared to that of any other viral system (1). At the same time, the study of their properties is progressing at such a pace that we may reasonably hope to approach a complete understanding of their structure and functions within a not too distant future.

Moreover, in recent years, a number of features concerning the organisation of RNA phage replication have emerged which could be of importance for other viruses as well. Three of these features, which are in fact interrelated, will be discussed in detail:

a) Economy of genetic information: The finding that most or all of the virus-specific components have evolved to carry out more than one function in the course of the viral replication cycle.

b) The ability of the viral system to not only take advantage of the synthetic machinery of the cell, but also use a number of host components for specific viral functions which are apparently quite *unrelated* to the tasks these components perform in the host cell.

c) A relatively complex regulatory network, where synthesis and

function of each viral component is influenced by interactions with other components.

In our laboratory, work in this field has been concentrated in the last several years on the phage Qβ. This phage is quite similar, although serologically unrelated, to the other members of the group of small RNA phages, like MS2, R17 and f2.

II. The Viral Particle and Its Reproductive Cycle

Qβ is a small icosahedral particle of approximately 4.2 million molecular weight. It consists of four molecular components, namely:

a) A single-stranded RNA of 4200 to 4500 nucleotides.

b) The major capsid polypeptide ("coat protein"), which has a molecular weight of 14,000. The viral particle contains about 180 copies of this protein.

c) The maturation protein ("A_2-protein"), with a molecular weight of approximately 44,000. Each particle contains one molecule of this protein, whose function is necessary for the process of infection.

d) A second minor capsid protein ("A_1-protein"), whose function is not known. Its molecular weight is about 38,000 and each viral particle contains 2-5 copies of it.

The reproductive cycle of Qβ, as that of other RNA phages, consists of the following stages:

a) Particles infect male-specific *E. coli* by attaching to the pili and transferring their RNA into the interior of the cell.

b) The viral RNA serves as messenger for the synthesis of the viral proteins, one of which is a subunit of a phage-specific RNA-dependent RNA polymerase ("replicase").

c) The polymerase uses the parental RNA as a template for the synthesis of complementary RNA strands ("minus strands") which in turn serve as templates for the synthesis of many new viral strands ("plus strands").

d) Both RNA and protein synthesis continue until large quantities of the particle components accumulate.

e) Self-assembly of the components leads to a large number of progeny particles (20-50,000).

f) The phage particles are released by cell lysis.

Since interest in our laboratory has been mainly focused on the expression and the replication of the viral genome, the further discussion will concentrate primarily on these aspects of viral reproduction.

III. Expression of the Viral Genome

A. The Number of Cistrons

Complementation analysis of mutants of phage Qβ have shown the existence of 3 cistrons on the viral RNA (2). Studies on phage-specific protein synthesis *in vivo* and *in vitro* have allowed to correlate these with the viral coat protein, the β subunit of the viral replicase (MW 69,000) and the maturation (A_2) protein (3). The complete aminoacid sequence of the Qβ coat protein has been determined (4), but only a few N-terminal aminoacids are known of the other Qβ proteins.

The second minor particle protein mentioned above —the "A_1-protein"— has an unusual genetic origin. The findings that (i) amber mutants in the coat cistron do not produce A_1, and (ii) UGA suppressors both *in vivo* and *in vitro* increase the production of A_1 (3, 5), led to the hypothesis that this protein might be the product of a readthrough process, arising when translation of the coat cistron continues beyond the coat termination site. Recently this explanation has gained convincing support by the demonstration that the N-terminal aminoacid sequence of A_1 is identical to that of the coat protein (5, 6). The question as to why a fourth complementation group corresponding to the carboxyterminal two-thirds of the A_1 protein is not found cannot be answered at present; either this region of the genome does not give rise to suppressible nonsense mutations or the readthrough protein might be without an essential function.

B. The Ribosomal Binding sites

If Qβ RNA is bound *in vitro* to *E. coli* ribosomes in the presence of formylmethionly-tRNA and GTP, a translational initiation complex results. The site of interaction of the ribosome with the RNA can be analyzed by using ^{32}P-labelled phage RNA and degrading the initiation complex with ribonuclease. Under adequate conditions a fragment of ^{32}P-RNA which is protected from nucleolytic degradation by its interaction with the ribosome can thus be isolated. After purification of the fragment, its sequence can be determined by standard techniques. By this approach the nucleotide sequences of the ribosomal initiation sites of all three Qβ cistrons have been obtained (7-10). They are shown in figure 1, along with the analogous sequences on the RNA of phage R17 (11). In all cases an AUG initiator codon is found near the middle of the fragment, followed by the triplets coding for the first few aminoacids of the corresponding protein.

The three binding sites on Qβ RNA have very different properties with respect to their capacity for binding ribosomes. If intact, native Qβ

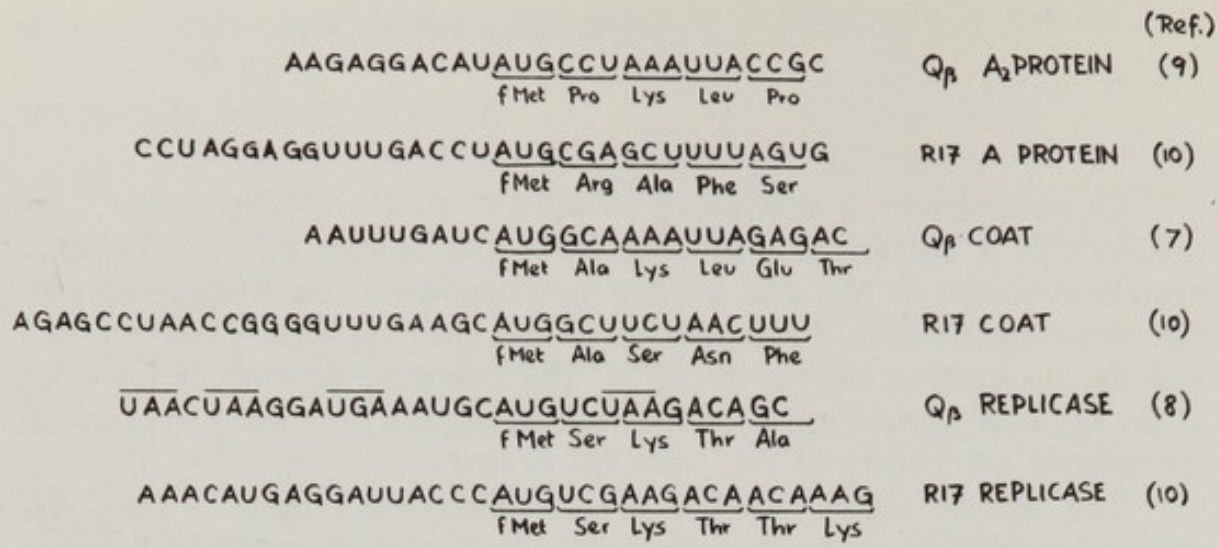

FIG. 1. Nucleotide sequences of the ribosome binding sites of Qβ and R17 RNA. Each segment contains a formylmethionine initiator codon AUG followed by the triplets coding for the first few aminoacids of the corresponding protein. The binding site of the replicase cistron of Qβ contains 4 possible nonsense codons which are marked by horizontal bars above the triplet.

RNA is used, the binding of the ribosome takes place predominantly at the coat protein initiation site as judged by the protection experiments described above. Fragmentation of the RNA (8, 10) or other treatments affecting the secondary structure of the RNA (12) leads to a substantial increase in the yield of the replicase cistron initiation site, whereas the maturation protein initiation site has up to now only been obtained with nascent RNA strands, i.e., short fragments containing the 5' end (9). From this, the conclusion has been drawn that the secondary structure of the phage RNA must play an important role in regulating the initiation of synthesis of the various gene products, a point to which we shall return below.

C. The Map of the Qβ Genome

The arrangement of the three cistrons relative to each other on the Qβ genome could be elucidated by biochemical mapping techniques.

The maturation protein initiation triplet was found to be located at position 62 from the 5' end of the RNA, a region which had been sequenced earlier by the synchronized labelling technique developed by BILLETER *et al.* (13). The maturation protein cistron thus is the one closest to the 5' terminus.

In order to locate the coat cistron binding site, a collection of Qβ RNA molecules labelled for different lengths from the 5' terminus was synthesized *in vitro* (fig. 2) (14). Ribosome binding experiments yielded radioactive coat cistron binding site only if the Qβ RNA was labelled to a

region between the 1100th and the 1400th nucleotide from the 5' terminus. This places the coat protein cistron at an interior position of the RNA, and, since the gene for the maturation protein (MW 42-44,000) can be estimated to be about 1100 nucleotides long, the coat gene is probably adjacent to it.

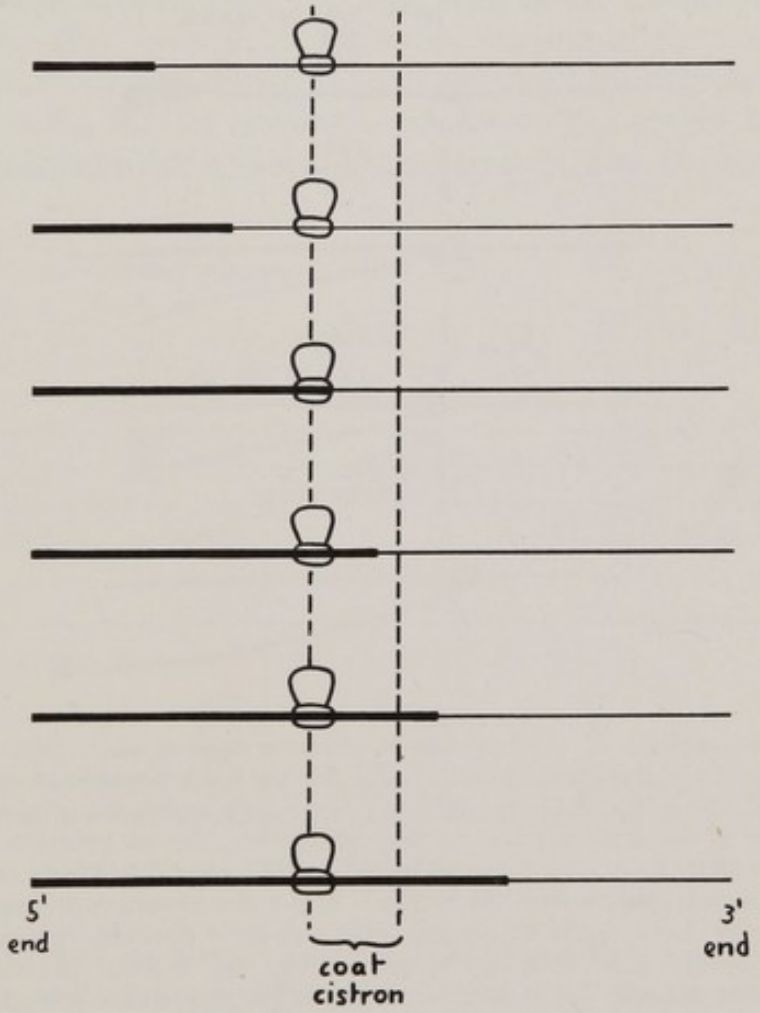

FIG. 2. Location of the ribosomal binding site of the coat cistron of Qβ RNA. Using ^{32}P-labelled substrates several preparations of labelled Qβ RNA segments extending to different lengths from the 5' terminus were synthesized in time-limited synchronized reactions by Qβ replicase (14). After withdrawal of aliquots for size determination by sucrose gradient analysis, the labelled segments were elongated using non-radioactive triphosphates. This procedure resulted in a collection of full-length Qβ RNA strands labelled to different, defined extents from the 5' end. Ribosomes were bound to these RNA preparations and the RNA segment corresponding to the binding site was isolated and characterized. Since only molecules labelled beyond the beginning of the coat protein cistron could yield ^{32}P-labelled binding site, this site can be located (14).

The relative order of the coat and the replicase cistrons was determined in a separate experiment. A ribosome was bound to Qβ RNA and the RNA-ribosome complex was used as a template for Qβ replicase, with unlabelled ribonucleoside triphosphates as substrates (fig. 3). Elongation ceased when replicase reached the ribosome. The ribosome was

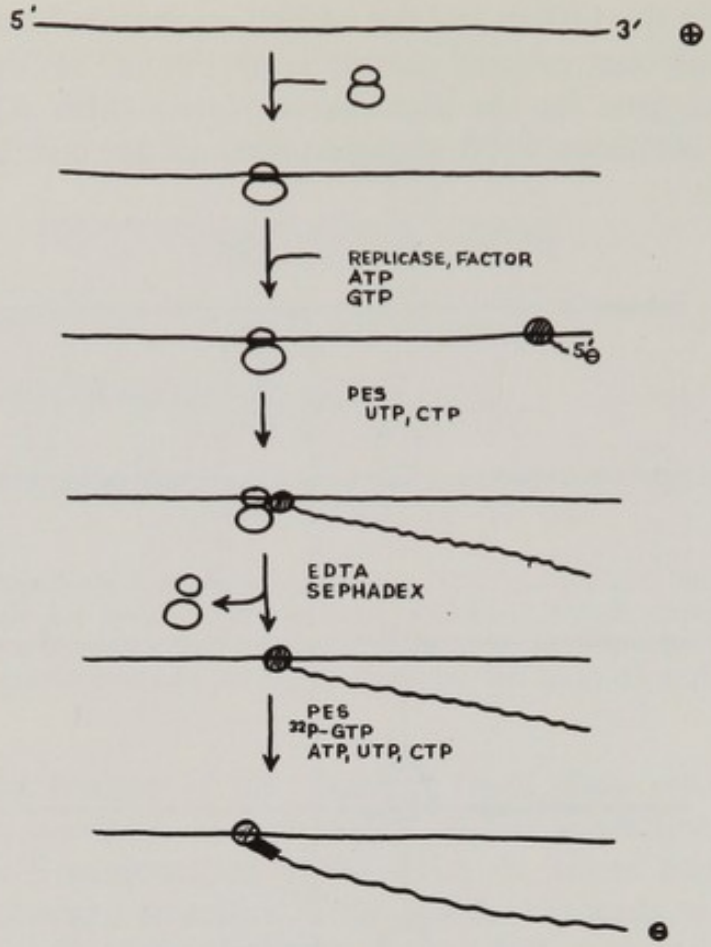

FIG. 3. Resynchronization of Qβ replicase at ribosome binding sites: Determination of the relative order of coat and replicase cistrons (15, 22). The Qβ RNA-ribosome complex is incubated with Qβ replicase, host factor, ATP and GTP to give an early replicating complex. Polyethylene sulfonate is added to inactivate any free enzyme and thereby prevent initiation during the later phase of the experiment. Elongation is started by adding UTP and CTP. After 5 min at 37° EDTA is added to remove the ribosome from the complex. EDTA and substrates are removed by Sephadex chromatography. The complex is then incubated with the 4 standard triphosphates, of which one or all are α-labelled with ^{32}P at high specific activity, for 15 sec at 20°, to allow synthesis of a labelled segment of about 100 nucleotides in length. The product could be separated into two species. In the longer RNA (length 3000 nucleotides), the labelled segment contained sequences complementary to the ribosomal binding site of the coat cistron; in the shorter product (200 nucleotides), the radioactive segment was complementary to the ribosomal binding site of the replicase cistron.

then removed from the RNA by treatment with EDTA and the replicating complex was separated from substrates and EDTA by gel filtration. On addition of radioactive triphosphates, synchronized synthesis ensued and labelled minus strand segments were produced. Sucrose gradient centrifugation or polyacrylamide gel electrophoresis separated two products, a major one of about 3000 and a minor one of about 2000 nucleotides length. Sequence analysis revealed that in the longer RNA the labelled segment was complementary to the coat protein initiation site; in the shorter product, the label was found to be contained in a sequence com-

 plementary to the replicase cistron initiation site (15). These results show

that while the majority of the ribosomes bind to the coat cistron initiation site, some bind instead to the initiation site of the replicase cistron. Furthermore, it establishes the location of the replicase cistron between the coat cistron and the 3' end, suggesting a distance of about 1000 nucleotides between the two ribosomal binding sites.

We are now able to draw a genetic map of Qβ phage (fig. 4). At the 5' terminus we first have an extracistronic region of 61 nucleotides, the function of which is not known. This is followed by approximately 1100 nucleotides coding for the maturation protein. The length of the region between the cistrons for the maturation protein and the coat protein is

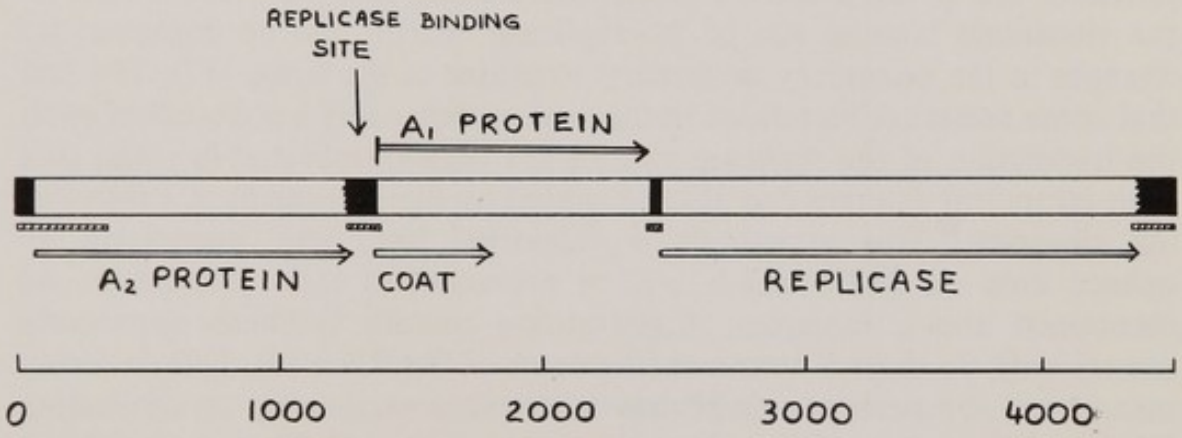

FIG. 4. The map of the Qβ genome. The noncoding regions are given as dark areas. The regions of known nucleotide sequence are indicated as hatched bars below the map. For further explanations see text.

not known. As will be shown below, a sequence of 97 nucleotides to the left of the coat initiation site contains a number of nonsense codons in all three reading frames (cf. fig. 6), but since the carboxy terminal aminoacid sequence of the maturation protein is not known it cannot be decided which of these serves as termination signal. The region between the initiation sites for the coat and the replicase cistrons comprises about 1000 nucleotides. Of these, 400 code for the coat protein, while the other 600 nucleotides are thought to be translated into the carboxyterminal two-thirds of the readthrough protein. The molecular weight of the readthrough protein (38,000) is indeed compatible with about 1000 coding nucleotides. Furthermore, inspection of the RNA sequence near the replicase cistron initiation site reveals the presence of terminator codons in all three reading frames (cf. fig. 1), so that an out-of-phase reading into the replicase cistron during readthrough protein synthesis is excluded (10). The replicase cistron (about 2000 nucleotides) is followed by an extracistronic region of not less than 61 nucleotides (16).

D. Regulation of Protein Synthesis

In vivo, the phage cistrons are translated to widely differing extents, which also vary with the time after infection. Coat is the major product of protein synthesis throughout the infectious cycle. Replicase activity appears as early as 10 min after infection but further synthesis of the enzyme ceases 15 min later (3). Maturation protein is synthesized only in small amounts, with a maximum at about 30 min after infection (17).

Thus obviously the production of the viral proteins is a regulated process. We have already mentioned that a major factor in this regulatory system seems to be the structure of the RNA. On native RNA the coat initiation site is the preferred ribosomal binding site (7). Accessibility of the ribosomal binding site of the replicase cistron can be improved by changes in the secondary or tertiary structure of the RNA (12). The fact that some nonsense mutations in the coat protein exert a polar effect upon the translation of the replicase cistron has been interpreted to mean that such structural changes can also be caused by the movement of a translating ribosome. This hypothesis is supported by FIERS' impressive sequence data on MS2, which will be presented at this symposium. As mentioned above, initiation of maturation protein synthesis apparently occurs only on short 5' terminal fragments of Qβ RNA (9). This probably means that synthesis of this protein takes place exclusively on replicating RNA complexes containing short nascent strands (18). Thus, a viral RNA molecule is available for only a very short time as messenger for the maturation protein.

In addition to the regulating mechanisms built into the structure of the RNA itself, phage-specific protein synthesis is also subject to at least two systems of translational repression. One of these seems to be important for the transition of the viral RNA from its role as a messenger to that of a template for its own replication. Shortly after infection, the phage RNA is present as a polysome, with ribosomes travelling in the 5' to 3' direction. In replication, the viral polymerase advances along the template in the 3' to 5' direction (19, 20), i.e., on a collision course with translating ribosomes. Since replicase cannot dislodge ribosomes bound to Qβ RNA (21), a special mechanism must exist to free the parental RNA of ribosomes and render it competent as template for replication.

A clue to the resolution of this problem was obtained by the finding, shown in figure 5b, that Qβ replicase strongly and specifically inhibits binding of ribosomes to Qβ RNA (21). Elongation and termination of initiated polypeptide chains are not affected, however, so that this inhibition leads to a rapid dismantling of the polysome (22). The mechanism of this inhibition was elucidated by the demonstration that replicase is capable of binding to the same region of the RNA as the ribosome. It had been known for a long time that Qβ replicase binds Qβ RNA rather strongly,

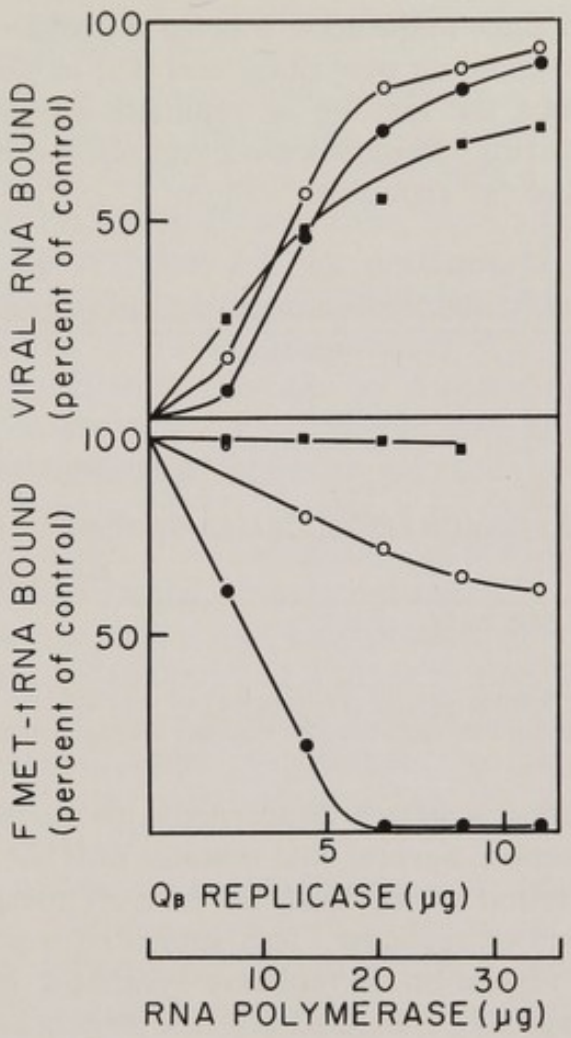

FIG. 5 (a). Binding of labelled viral RNA to Qβ replicase or DNA-dependent RNA polymerase as measured by the Millipore filter assay. (b) Effect of Qβ replicase or DNA-dependent RNA polymerase on phage RNA-dependent binding of [^{3}H] formyl-methionyl-tRNA to ribosomes. Filled circles= Qβ replicase and Qβ RNA; open circles= Qβ replicase and R17 RNA; squares= DNA-dependent RNA polymerase and Qβ RNA (21).

a reaction which can be measured by a simple nitrocellulose filter assay (19) (see figure 5a).

In order to determine on what segment of the RNA this interaction takes place, binding complex containing ^{32}P-labelled Qβ RNA was subjected to limited digestion by ribonuclease T_1 and recovered by filtration through nitrocellulose filters (23). The ^{32}P-RNA fragments were extracted and separated by polyacrylamide gel electrophoresis. Sequence analysis showed that most (but not all) of these fragments originated from a single site, comprising 100 nucleotides, which partly overlaps the ribosomal binding site at the coat cistron (fig. 6). Thus, replicase, which binds to Qβ RNA tightly and far more rapidly than the ribosome, competes with the latter for the same site on the RNA. Since, as mentioned above, translation of the replicase cistron is dependent on the synthesis

of coat protein, and since maturation protein is probably not synthesized on complete RNA chains, no new ribosomes will be able to attach to Qβ RNA any more once the binding of replicase has taken place. Thus, after the last synthesizing ribosomes have run off, the RNA is free for its function as a template for replicase.

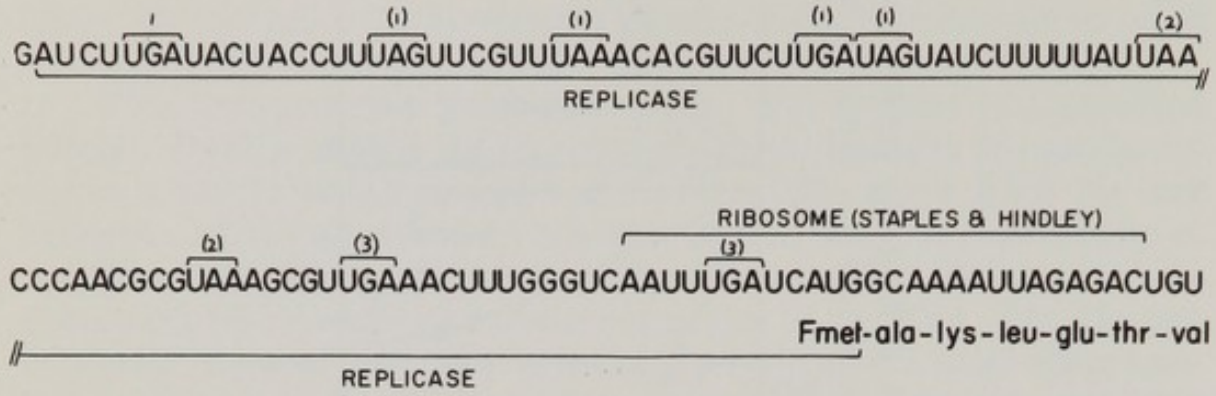

FIG. 6. The overlapping binding sites of replicase and of the ribosome at the beginning of the coat cistron. Horizontal brackets above the sequence indicate nonsense triplets (23).

Another regulation system is concerned with the turn-off of replicase synthesis late in infection. Several coat mutants of RNA phages, including Qβ (2), have been found which lack this turn-off mechanism and accumulate large amounts of replicase. This suggested a possible role of the coat protein in the regulation of replicase synthesis. Indeed, addition of coat protein to a phage RNA-directed cell-free protein synthesizing system was found to inhibit synthesis of the replicase gene product (24). In a binding experiment similar to the one discussed for Qβ replicase, BERNARDI and SPAHR (25) recently isolated a piece of R17 RNA which is protected from RNase digestion by the binding of R17 coat protein. This segment overlaps with the ribosomal binding site for the replicase cistron. The analogous experiment in the Qβ system has not yet been carried out.

IV. Replication of the Viral RNA

The broad outline of Qβ-specific RNA synthesis has been established by the early work in the laboratories of SPIEGELMAN (20), AUGUST (19) and WEISSMANN (26). After infection by Qβ, a new RNA polymerase, Qβ replicase, appears in *E. coli* cells, from which it can be extracted and purified. It is able to use Qβ RNA as template and, in a first step, synthesizes an RNA strand complementary to it. This minus strand does not, during replication, exist as a double-helical structure together with the plus strand. Instead, it occurs as a free single-strand which immediately after its completion is used by the replicase as a template for the synthesis

of viral plus-strands. This was the first system allowing net synthesis of infectious viral nucleic acid *in vitro* (27).

The enzyme shows a remarkable specificity with regard to its template RNA: Besides Qβ plus and minus strand RNA, only some species of small RNAs known as "Qβ variants" (20) and as "6S RNA" (28) are replicated. In addition, poly C and other synthetic polymers rich in cytidine are used as templates for the synthesis of the complementary strand (19). No other cellular or viral RNA has been found to be active, and other homopolymers are actually inhibitory to the enzyme.

As was discovered some time ago by AUGUST and co-workers (19), purified replicase, in order to be able to replicate Qβ RNA, requires an additional protein component which is supplied by the host cell. The presence of this "host factor" is necessary for the *in vitro* synthesis of minus strands from a plus strand template, but not for the reverse process nor for the reactions with the other templates.

The pure enzyme consists of four protein subunits, called α (MW 74,000), β (69,000), γ (45,000) and δ (35,000) (29, 30). It was immediately clear that not all of these proteins, which have a combined molecular weight of over 200,000, could be coded for by the phage genome, and differential labelling experiments showed that the β subunit is the only phage specified polypeptide.

The normal cellular functions of the host-specified subunits were elucidated recently. γ and δ have been identified by BLUMENTHAL *et al.* (31) as the protein synthesis elongation factors T_u and T_s. The α subunit has been shown by GRONER *et al.* (32) to be identical with factor i, a protein capable of inhibiting polypeptide chain initiation at certain cistrons, e.g. at the coat but not at the replicase cistron of phage RNAs. The cellular function of the "host factor" has not yet been identified. So far, the specific roles of the four host polypeptides and the replicase gene product in the phage RNA replication system remain unkown. Certainly, one or more of these components must be involved in the mechanism of the specific RNA recognition process.

As outlined above, a binding site of Qβ replicase on Qβ RNA has been isolated and found to be overlapping with the coat cistron ribosomal binding site. There are, however, reasons to suspect that this binding site does not play an essential role in template recognition and replication but rather, that it evolved specifically for the repression mechanism discussed above. One reason is the observation, mentioned before, that Qβ RNA which carries a ribosome bound to the coat initiation site is still an active template for replicase. Another argument is based on some recent results obtained by SCHWYZER *et al.* (33) in an investigation of the capacity of Qβ fragments to serve as templates for Qβ replicase. The data clearly show that contrary to earlier belief, fragmented Qβ RNA is still fully active as a template provided it has a size of not less than about 20 S, i.e.

approximately 2000 nucleotides. It was shown further that only fragments containing the original 3' termini serve as templates. Since the coat cistron binding site, which has been shown to interact strongly with replicase, is at a distance of about 3000 nucleotides from the 3' end, its presence on the RNA is apparently not needed for template activity. Rather, loss of template activity on fragmentation seems to be associated with the loss of a site near the middle of Qβ RNA.

Among the products obtained following digestion of replicase-RNA complexes a few fragments had in fact been isolated which did not originate from the coat cistron region. The yield of some of these fragments can be improved by choosing different reaction conditions and we are presently investigating their origin and their sequences.

An unexpected aspect of these binding experiments is the fact that under no conditions have we been able to find a fragment from the 3' terminus of Qβ RNA, not even after RNA synthesis had been initiated. Since of necessity some kind of interaction must occur between replicase and this part of the RNA, we have to conclude that this interaction is probably not tight enough to protect this segment against RNase digestion.

The (hypothetical) tight binding site discussed above, located near the middle of the RNA, might serve not only to increase the overall affinity for replicase but also to position the 3' end favorably for the initiation of synthesis. The specificity of template recognition would thus be in part due to the overall conformation of the RNA. R17 RNA, although its 3' terminal sequence is not very different from the one of Qβ RNA (1), is no template presumably because it binds Qβ RNA strongly at a site which does not allow correct positioning of the 3' terminus. The fact that poly C does serve as template, although at a much higher molar concentration than Qβ RNA, could be explained by considering poly C to be an analog of the 3' end of Qβ RNA, a region of the RNA which, as mentioned above, probably has a relatively low affinity for replicase.

Recently a replicase species lacking subunit α has been isolated in our laboratory (34). It has the interesting property that its synthetic activity with Qβ plus strands as template is considerable decreased as compared to the holoenzyme, whereas with minus strands and poly C its activity is unchanged. The residual plus strand activity can be further reduced by increasing the ionic strength. The binding interaction between Qβ RNA and α-less replicase was found to be salt sensitive as well (R. I. KAMEN and H. WEBER, unpublished results). Digestion of these α-less complexes with T_1 ribonuclease yields less protein-bound RNA material as compared to holoenzyme complexes, and no defined fragments could be isolated. Thus, it seems likely that the α subunit, which is a host protein, plays an important role in the template binding and recognition process.

V. Conclusion

From all these results (and from many other which could not be included) Qβ emerges as a surprisingly sophisticated creature. As mentioned at the outset, the small size of their genome has forced the RNA phages to organize their replication in an extremely economical fashion. The viral RNA has a variety of functions. Not only is it a structural component of the viral particle but it also serves as a template for replication and as a messenger subject to multiple translational controls. The phage proteins have subsidiary regulatory roles in addition to their apparent main function. A number of host proteins has been recruited by the phage components to take part in the replication of the viral RNA, in functions seeming completely different from the normal activities of these proteins.

There are still many conspicuous gaps in our knowledge of RNA phage replication, particularly regarding the mechanism of interaction between the molecules involved.

However, from the sophistication of these relatively simple systems we may already anticipate the degree of complexity of animal viruses.

VI. Acknowledgments

The unpublished work from our laboratory cited in this paper was supported by the Schweizerische Nationalfonds No. 3506 and the Jane Coffin Childs Memorial Fund No. 243.

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STRUCTURE AND FUNCTION OF THE RNA OF BACTERIOPHAGE MS2

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CONTENTS

Structure and function of the RNA of bacteriophage MS2

I. Introduction

The RNA-bacteriophages have been studied very intensively since their discovery in 1961 (LOEB and ZINDER, 1961). Indeed they provide a relatively simple model system, not only for studying basic virological questions, such as viral RNA replication and physiology of the phage-infected cell, but also for solving fundamental and very general questions such as translation and even gene control mechanisms.

The genome of the bacteriophage MS2 (or R17 or f2, which are virtually identical) contains only three genes coding for the A-protein, the coat protein and the RNA-polymerase or replicase subunit (fig. 1).

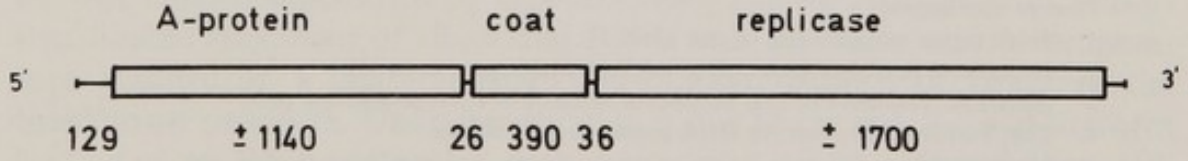

FIG. 1. The genome of bacteriophage MS2. The numbers refer to map distances in nucleotide units.

Approximately 30 min after infection the host cell lyses resulting in the release of over 10,000 virus particles (fig. 2). Each virion consists of the RNA enclosed by a shell of 180 coat protein molecules and one A-protein molecule. The latter is needed for adsorption.

But the viral RNA is a much more sophisticated molecule than a mere linear information tape. Indeed, it fulfills a variety of functions in translation, which is intricately controlled, in replication, in virus assembly, and in processes required to impose itself on the host cell metabolism. The

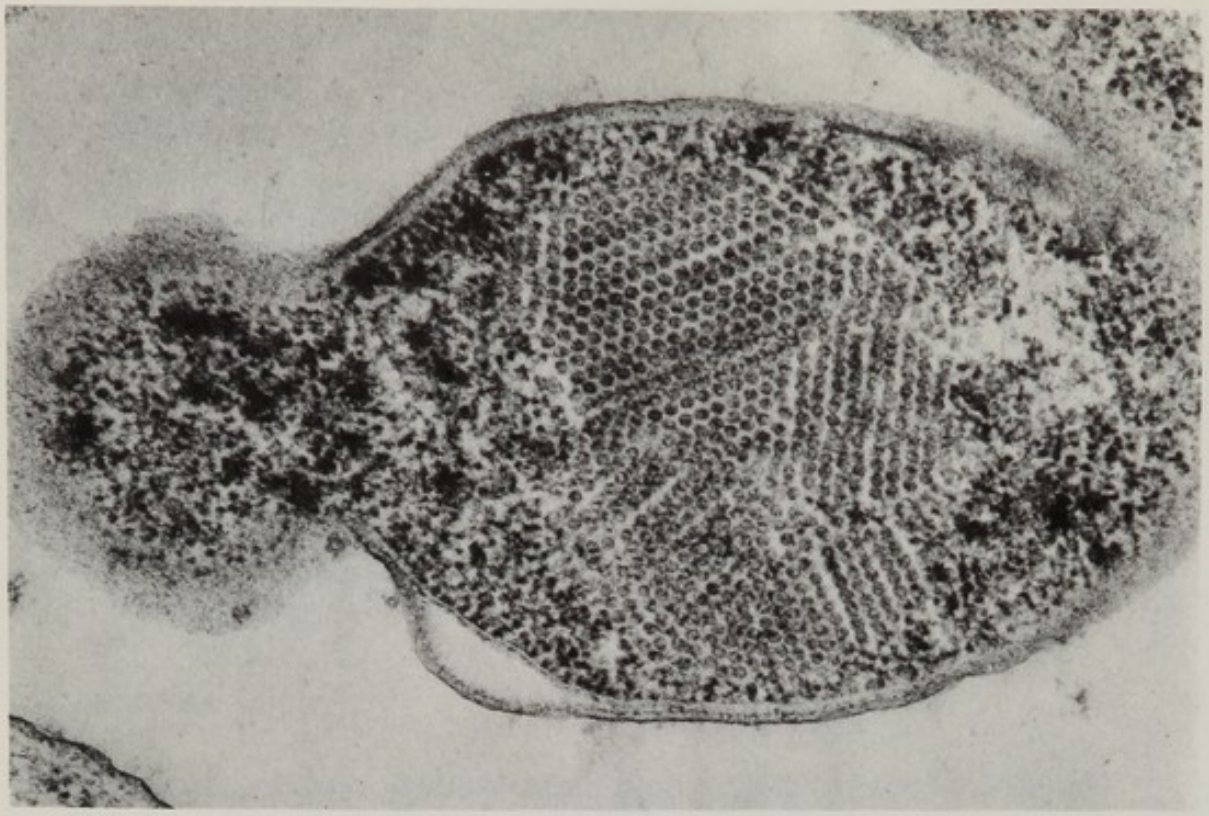

FIG. 2. Electron microscopy of a lysing, infected cell. The plate shows a lysing *Escherichia coli* C3000 cell, 180 min after infection. (The infection was at high cell density such that lysis was retarded.) The bacterium is loaded with viruses assembled in pseudocrystalline arrangements; the content streams out the broken cell wall. Uranylacetate-glutaraldehyde fixation (102,000×). (Kindly provided by C. MEYVISCH, H. TEUCHY and M. VAN MONTAGU.)

Table I. Functions of the bacteriophage RNA.

TRANSLATION

- Contains information for the amino acid sequence of three proteins.
- Control mechanisms:
  1. Much higher initiation rate of coat gene.
  2. Initiation of A-protein gene only possible on chains statu nascendi.
  3. (Polarity): translation of coat gene opens initiation at RNA-polymerase gene.
  4. Coat protein is repressor for RNA-polymerase translation.
  5. Modulation?

REPLICATION

- Specific recognition (of the 3'-terminal region?) of the *plus* strand by the RNA polymerase complex.
- Internal binding by the RNA polymerase complex in order to strip off ribosomes[1].
- One (or two?) cellular factors bind to *plus* strand templates and are required for replication.[1]
- Specific recognition (of the 3'-terminal region) of the *minus* strand by the RNA-polymerase complex.

OTHERS FUNCTIONS

- Viral RNA is metabolically stable.
- (Specific) interaction with the A-protein.
- Interactions leading to encapsulation.

[1]These processes have sofar only been observed with the bacteriophage Qβ-system.

known functions are summarized in table I, but the list is probably not yet complete. Most of these involve highly specific interactions, with ribosomes, or with host factors, or with virus-induced proteins, etc. Some of these interactions must also occur with the minus strand (RNA-polymerase), but other have to be excluded (ribosome binding, encapsulation). No doubt many of these phenomena will have to be explained not only in terms of primary structure, but also of secondary and even tertiary conformation. As the latter, however, are dictated by the former, we started out to determine the nucleotide sequence of MS2 RNA.

II. Experimental Approaches to the Determinaion of the Primary Nucleotide Sequence

Our earlier work had led to the isolation and the characterization of the terminal oligonucleotides from complete enzymatic digests of ^{32}P-labeled MS2 RNA (FIERS *et al.*, 1969). These results showed that translation of the phage genome does not start at the physical 5'-end nor does it continue as far as the 3'-end.

The elucidation of long internal regions of the RNA molecule, however, really meant another level of complexity. Indeed although MS2 is a small virus, its genome nevertheless consists of 3,500 monomers, which have to be ordered! Moreover, compared to amino acid sequence work, nucleotide sequence has the disadvantage that there are only 4 different monomers with rather similar chemical properties. This means that polynucleotides are usually harder to distinguish and harder to fractionate than peptides.

But fortunately, two innovations really provided a breakthrough. On the one hand it was shown by GOULD (1966) and by MCPHIE *et al.* (1966) that limited treatment of ribosomal RNA with nucleases at low temperature resulted in a characteristic, non-random pattern of large, partial breakdown products. Undoubtedly, this is due to the specific and uniform folding of the RNA-molecules, such that some sites are especially exposed. On the other hand the newly introduced electrophoresis on polyacrylamide gel technique opened up much enhanced possibilities for high-resolution separation of polynucleotides. Indeed it was soon found that also with viral RNA limited enzymatic digestion resulted in a specific collection of breakdown products, which can be separated in a number of discrete bands by gel electrophoresis (MIN JOU *et al.*, 1968; ADAMS *et al.*, 1969).

We have used mainly two types of digest, the first obtained at an enzyme to substrate ratio of 1 unit per 20 μg RNA followed by separation on a 12% gel, and the second at an enzyme to substrate ratio of 1 unit per 200 to 600 μg RNA followed by separation on a 6% gel (fig. 3). Both types of incubation were for 30 min at 0° C. Under the former conditions

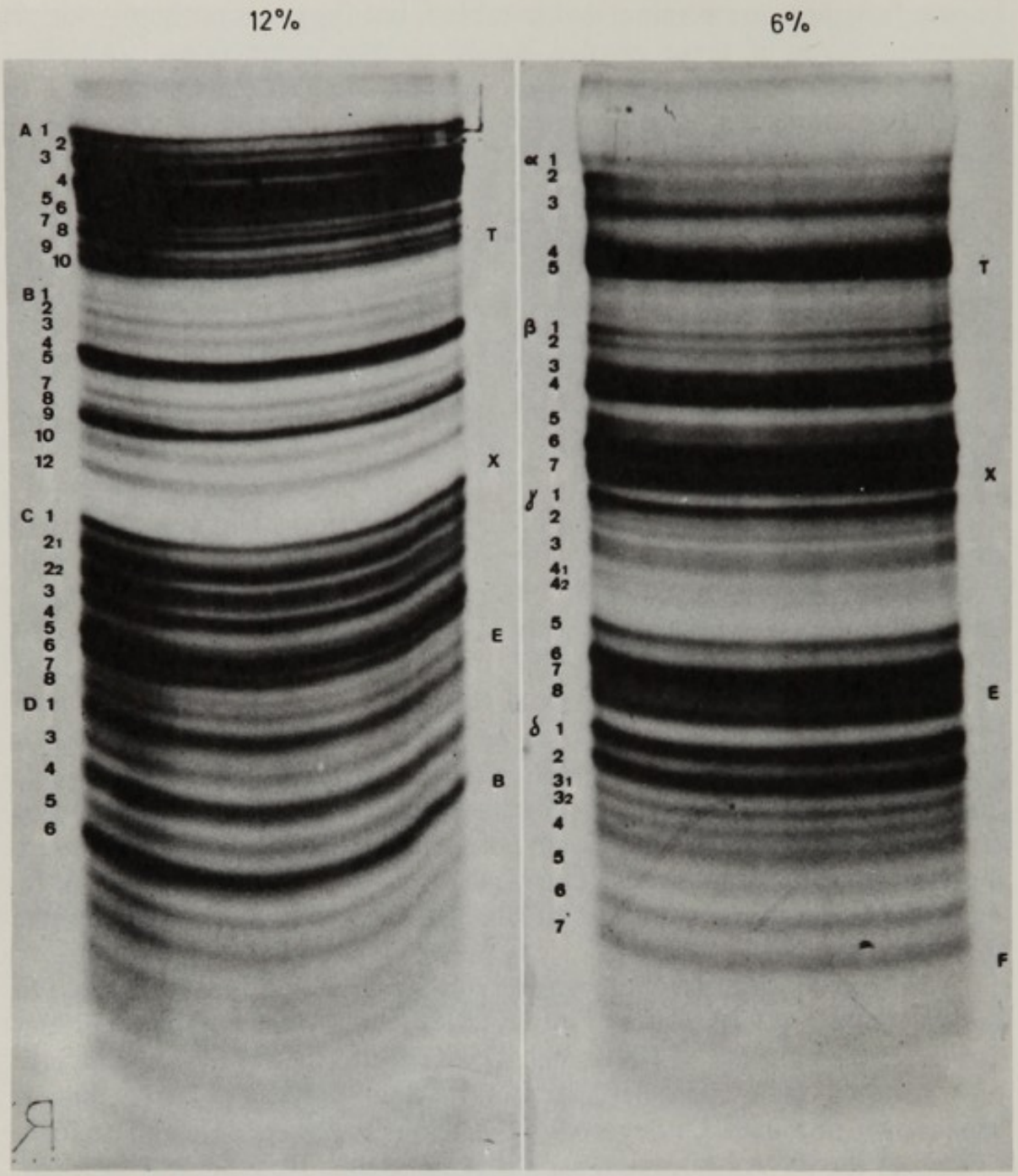

FIG. 3. Fractionation of a partial digest of ^{32}P-labeled MS2 RNA by electrophoresis on a slab gel. Partial digestion was carried out with ribonuclease T_1 at 0° C either at high (left) or at low (right) enzyme to substrate ratio. The former was separated on a 12% polyacrylamide gel and the latter on a 6%. A series of dyes, indicated on the right, are used as reference points in order to facilitate identification of the bands (T, trypan red; X, xylene cyanol FF; E, eosin; B, bromophenol blue; F, fluorescein). (DE WACHTER *et al.*, 1971b; DE WACHTER and FIERS, 1971).

mainly double-stranded regions (hairpins) remain intact, while under the latter conditions the same hairpins have single-stranded tails attached to both their 5' and their 3'-ends. Both patterns have reproducibly been obtained for almost three years. The bands in the 12% gel vary in chain

length from 30 to approximately 250; those in the 6% gel from 35 to approximately 700.

But each band is far from pure. In addition to one or more full length components, it contains complexes, held together by secondary binding forces. Fortunately, these can be fractionated by two-dimensional electrophoresis on polyacrylamide gel (De Wachter and Fiers, 1971, 1972). The first direction is at pH 3.5 in the presence of urea; under these conditions the complexes are dissociated and the fragments are separated according to size and to composition. The second dimension is at neutral pH, and now the mobility of the components is determined by their size and by their conformation. From most bands between a few to 20 pure fragments are obtained, which are now amenable to sequence determination according to the elegant methods developed by Sanger and collaborators (Sanger *et al.*, 1965; Adams, *et al.*, 1969).

The last step in the sequence determination consists in the ordering of the oligonucleotides which have been identified in a particular fragment. A renewed partial digestion can now be carried out, followed by separation on two-dimensional gels. Each product must again be fingerprinted by T_1 and pancreatic RNase for identification. Normally this would be an extremely time-consuming procedure, but here rescue came by the new technique of mapping on small polyethyleneimine plates (Southern and Mitchell, 1971; Contreras *et al.*, 1971).

Although real overlaps were seldom found between fragments in the primary digest, we often could group a particular set of fragments as being derived from the same region on the genome, e.g. a fragment A-E was isolated, but also fragments A-B, C-D and C-E. These related fragments are a considerable help in the nucleotide sequence elucidation of the particular region.

III. Untranslated Regions

A. The 5'-Terminal Leader Sequence

Since we know that MS2 RNA starts with the sequence pppG-G-G-U (De Wachter *et al.*, 1968), we could search for this group in longer fragments derived from the partial digests. Indeed, a series of these were found, which all started from the 5'-end and had chain lengths of 37, 74, 82, 89, 92, 102, 117 and 125 nucleotides, respectively (De Wachter *et al.*, 1971a, b). The complete sequence was established, and it turned out that it could be folded in a secondary structure model, as shown in fig. 4.

It was already clear that the 5'-terminal region was not translated, but the question became then: Where does the first cistron start? Fortu-

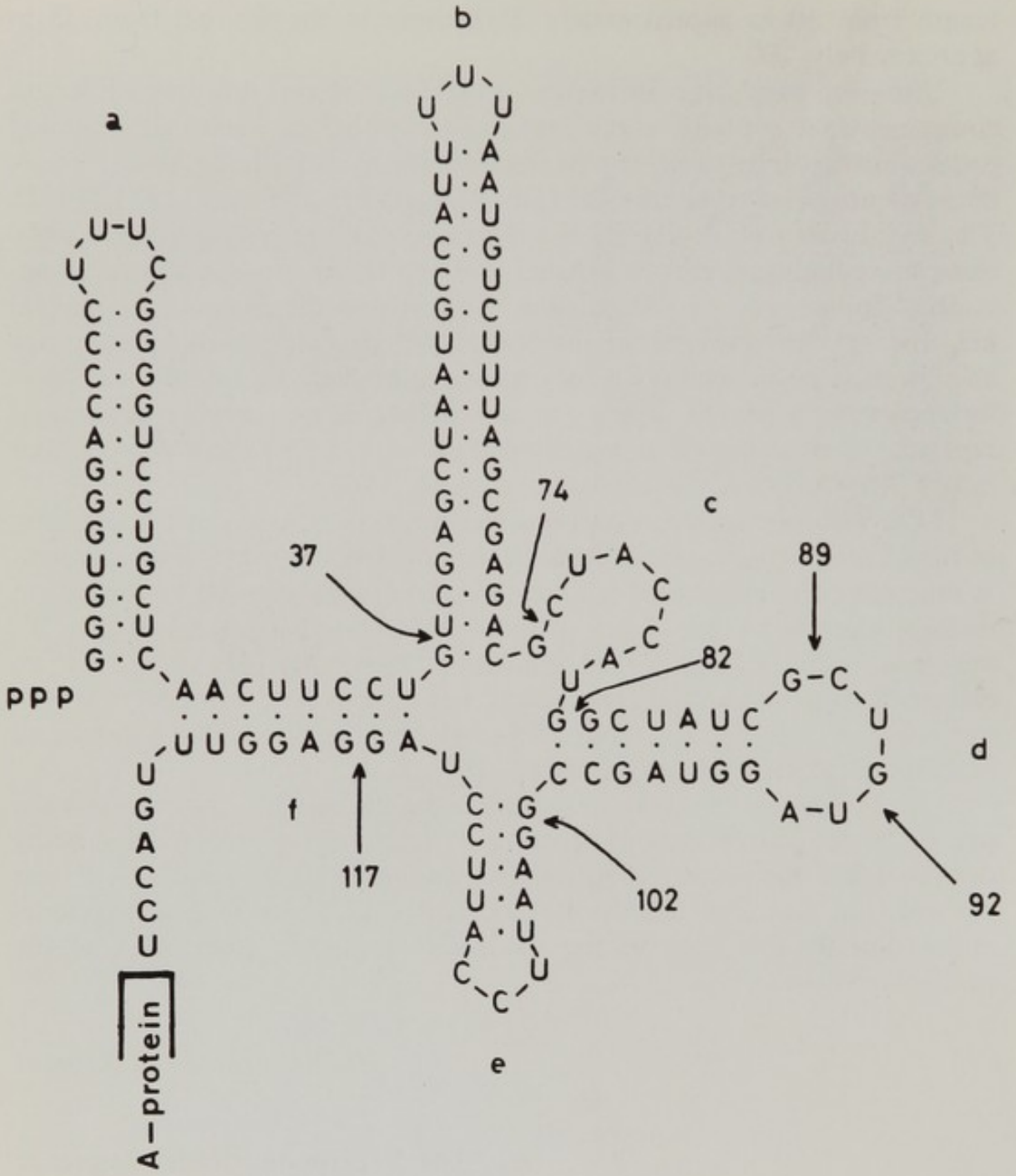

FIG. 4. The 5'-terminal sequence of MS2 RNA. The arrows point to sites easily split by ribonuclease T_1, and the numbers refer to the chain length of the isolated fragments which all start at the 5'-end (DE WACHTER *et al.*, 1971b; VOLCKAERT and FIERS, 1973).

nately, J. ARGETSINGER- STEITZ (1969) had already identified the regions surrounding the initiation codon of each phage cistron. She did this by binding ribosomes to the viral RNA under conditions leading to the formation of initiating complexes, followed by nuclease treatment and isolation of the labeled RNA pieces which had been protected by the ribosomes. We were happy to find an overlapping region between the 3'-

 end of our 5'-sequence and the ribosome binding region of the first cistron,

the A-protein. The overlap was 16 nucleotides in length, amply sufficient to prove that it is not due to a coincidence. In this way we could conclude that the first codon of the A-protein starts at nucleotide 130 from the 5'-end. Later work has shown that this initiating codon in MS2 is not AUG, as in R17, but is GUG (VOLCKAERT and FIERS, 1973).

ADAMS *et al.* (1972) have independently determined the 5'-terminal region of the closely related phage R17, up to position 117. It is most remarkable that they found a completely identical sequence, whereas in the translated regions for which sequences are available, 8 differences occur between MS2 and R17, or almost a 4% variation (FIERS *et al.*, 1971). Also, the spontaneous reversion frequency of phage mutants is generally high, which suggests that replication errors are rather common. Hence the stringent conservation of the nucleotide sequence at the untranslated 5'-terminal region indicates that a strong selection pressure is operative here. Most likely, this sequence exists as a specific 3-D structure, e.g. by further folding of the secondary structure model shown in fig. 4. What function this structure fulfills is not certain. It could be that the complement of this part, which is the 3'-end of the minus-strand and which is presumably folded in approximately the same way, acts as a recognition site for the viral RNA replication machinery.

It is noteworthy that the 5'-nucleotide sequence starts directly with a very tightly bound hairpin; possibly this offers a protection against exonucleolytic degradation.

B. The 3'-Terminal Sequence

The last 104 nucleotides of the MS2 RNA are shown in fig. 5. We had originally thought that perhaps the 3'-end would show similarities to the complement of the 5'end. Indeed, this would provide a simple explanation for the specific recognition of both the plus and the minus

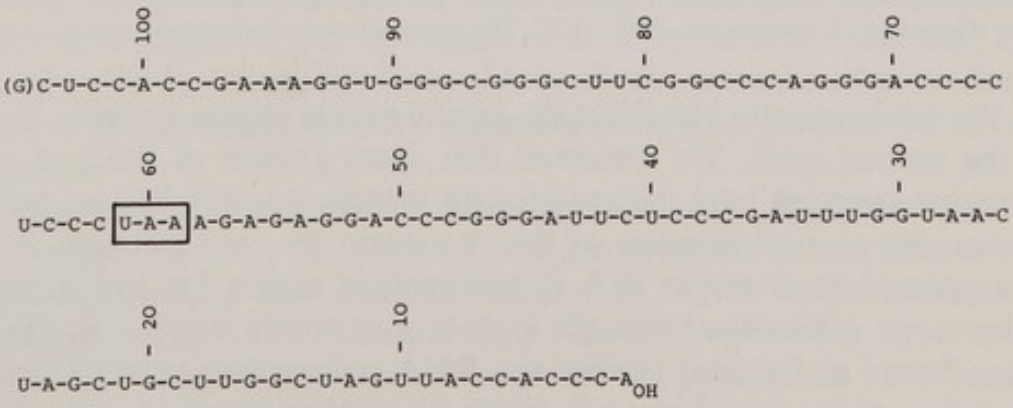

FIG. 5. The 3'-terminal sequence of MS2 RNA. The presumptive termination codon of the replicase gene is shown in the box (CONTRERAS *et al.*, 1971: VANDENBERGHE and FIERS, 1973).

strand by the same RNA-dependent RNA polymerase. However, the homology extends only over seven nucleotides, -C-C-A-C-C-C-A_3, and, although this may constitute an important handle to initiate replication, it seems hardly sufficient to explain the remarkable specificity exerted by the viral RNA polymerases in the recognition of their templates.

The last gene is the virus-coded RNA-polymerase polypeptide, and CAPECCHI and KLEIN (1970) have shown that the termination signal contains UAA, either alone or in a combination. We have some reason to believe that the UAA at 61 nucleotides from the 3'-end would correspond to this signal (VANDENBERGHE and FIERS, 1973).

C. The Intercistronic Regions

As the RNA genome contains three cistrons, there are two intercistronic divides or regions. NICHOLS (1970) could characterize a fragment from R17 RNA which contained the nucleotide sequence coding for the last 6 amino acids of the coat protein followed by the termination signal UAA-UAG. As the 3'-end of this fragment formed an overlap with the initiation region of the following gene, the RNA-polymerase, he could conclude that a segment 36 nucleotides in length, separates these two genes (fig. 6). Working with MS2, we found exactly the same intercistronic sequence, except for a single U to C transition.

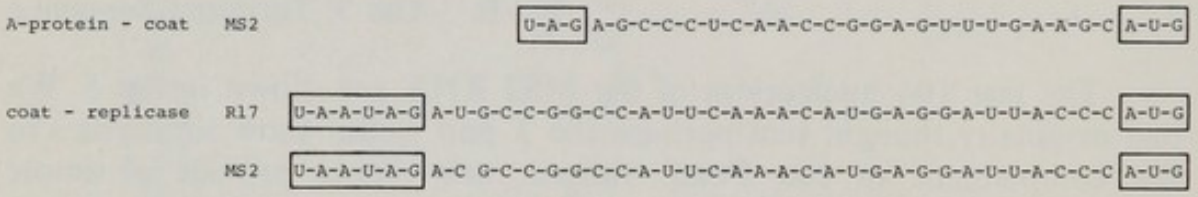

FIG. 6. The intercistronic regions. Top: region between the A-protein cistron and the coat cistron (CONTRERAS *et al.*, 1973). Bottom: region between the coat cistron and the RNA polymerase cistron (NICHOLS, 1970; MIN JOU *et al.*, 1972). The termination and initiation signals are enclosed.

We have recently identified the intercistronic region between the first and the second gene. We observed that upon growth of MS2 in amber suppressor-carrying host strains a fourth protein was synthesized and that this was due to prolongation of the A-protein by read-through. All evidence pointed to a single U-A-G termination codon for the A-protein. Furthermore, it was clear that the U-A-G stop codon was not in the same reading frame as the coat (unlike the RNA-polymerase cistron), and that termination of the fourth protein involved another stop codon, U-A-A or U-G-A (REMAUT and FIERS, 1972). We could elucidate the nucleotide sequence of 160 nucleotides preceding the coat gene, and close inspection

revealed that only one U-A-G sequence, 26 nucleotides before the initiating A-U-G of the coat, fulfilled all requirements and hence could be identified as the termination codon of the A-protein (fig. 6; CONTRERAS *et al.*, 1973). Amino acid sequence determinations by VAN DE KERCKHOVE *et al.* (1973) have confirmed this conclusion.

IV. The Coat Gene

A. The Primary Structure and a Model for the Secondary Interactions

The amino acid sequence of the coat protein of the phages f2 and R17 is known (WEBER and KONIGSBERG, 1967; WEBER, 1967), and the sequence for the MS2 coat protein was presumably identical to that of R17. By determining nucleotide sequence of fragments and comparing these with the known sequence of the R17 coat polypeptide, ADAMS *et al.* (1969) were the first to directly correlate a short sequence of genetic information with the sequence of the resulting gene product, the amino acid sequence in the corresponding region.

Working with MS2 RNA, we could identify similar regions, derived likewise from the MS2 coat protein gene (MIN JOU *et al.*, 1971). The first sequences to be isolated were all rather short and corresponded in fact to hairpins which had withstood the ribonuclease digestion at high enzyme to substrate ratio. Using milder digestion conditions and taking advantage of the high resolving power of the two-dimensional electrophoresis technique, we could isolate longer and longer fragments, which were all derived from the coat cistron. In fact, these most often correspond to previously identified hairpins, but prolonged at both ends by single-stranded tails. In this way we could finally deduce the complete nucleotide sequence of the gene (fig. 7; MIN JOU *et al.*, 1972). In fact three GpN links were always split by the T_1-ribonuclease, even under the mildest conditions used. Hence the continuity of the nucleotide sequence at these sites was based on the amino acid sequence. But by means of an enzyme with a different type of specificity we have recently been able to obtain the necessary overlaps and to prove the complete primary structure purely on the basis of nucleotide sequence.

Physico-chemical studies have revealed that approximately 63 to 82% of the bases in the phage RNA are involved in secondary interactions, base-pairing and stacking (MITRA *et al.*, 1963; BOEDTKER, 1967; ISENBERG *et al.*, 1971). Undoubtedly, this folding is very specific, which means that most if not all molecules in the population have largely an identical 3-D structure. Otherwise we would never have obtained the specific partial degradation patterns shown in fig. 3. The secondary structure is at least partially retained in the resolved bands, e.g. the band

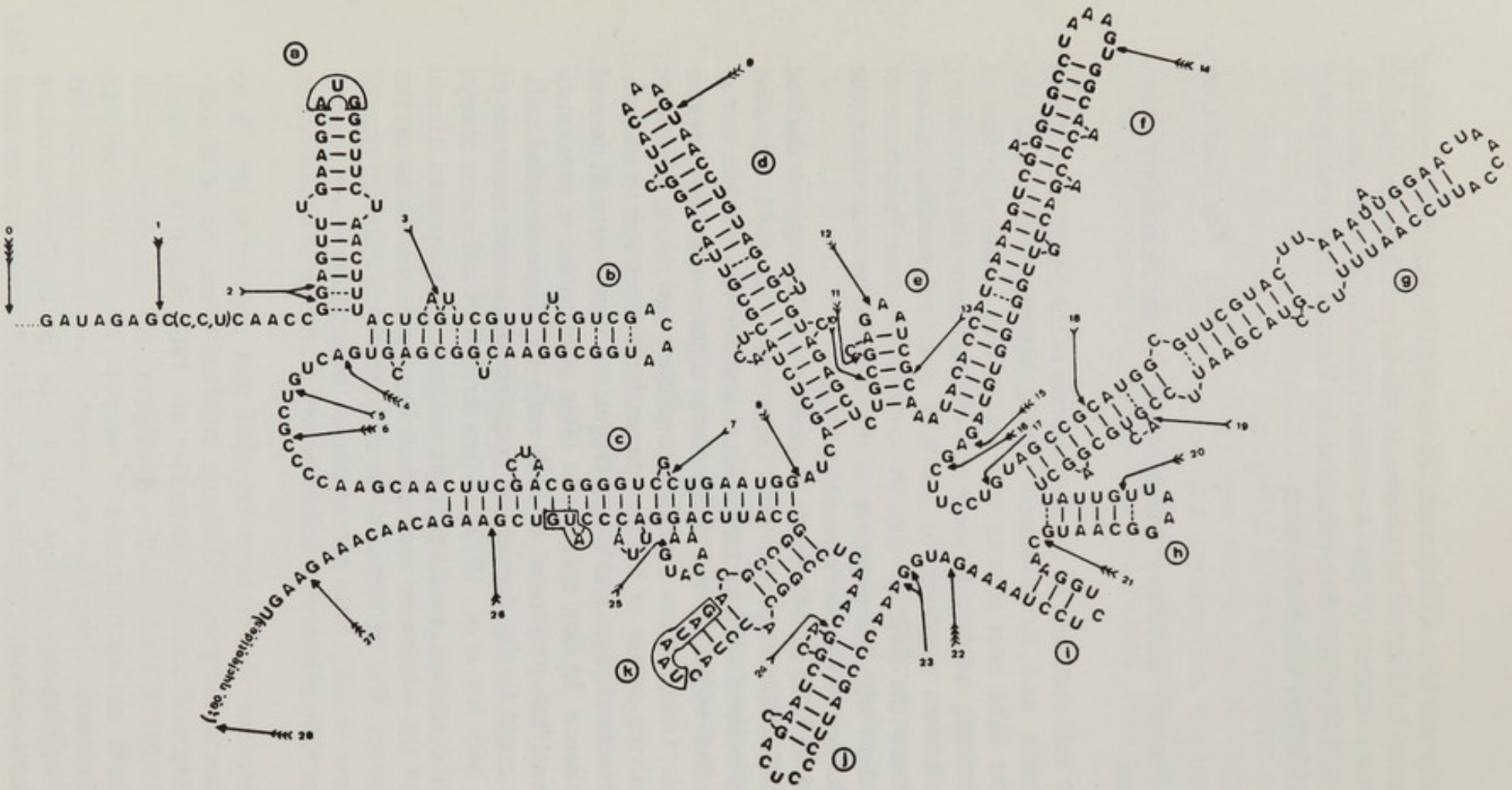

FIG. 7. "Flower"-model for the MS2 coat protein gene. The reading starts at arrow 0 and ends at arrow 28 in the following order: intercistronic region A-protein — —coat protein — —initiating A-U-G of the coat gene (enclosed) — —the coat gene — —the termination signal U-A-A-U-A-G of the coat gene (enclosed) — —the second intercistronic region — —initiating A-U-G (enclosed) of the last gene, the replicase or RNA-polymerase. The arrows point to bonds split by the ribonuclease T_1 in the partial digests, and the feathers are a measure of the susceptibility. GpN links indicated by four-feather arrows are always split (from MIN JOU *et al.*, 1972).

C5 contains both a hairpin of chain-length 61, as well as both separate arms of chain length 27 and 32, respectively (derived from the same hairpin but with a nick in the loop). The latter two must have been present as a complex for they moved in the gel like a component consisting of approximately 60 nucleotides. Many more examples of this type are known.

So, it seemed reasonable to start from these partial digestion products in order to construct a model for the secondary structure. In fragments derived from the more severe type of partial digest, most base pairs forming a hairpin can be directly derived from inspection of the primary structure. Nevertheless it is often possible to draw several models, which are similar in outline but which vary in detail. A choice can then be made on the basis of an estimate of the thermodynamic stability. TINOCO *et al.* (1971) have proposed a set of simple rules which permit evaluation of the relative stability of various pairing schemes, and hence selection of the most stable configuration. The next step was then to build out the model further by considering the fragments obtained in the milder partial digest and the interaction between some of these fragments as evidenced by their electrophoretic mobility. Again, estimates of thermodynamic stability were taken as a basis for choosing between alternatives. Finally, the model, shown in fig. 7 was derived. We have called it the "flower"-model as it consists of a stalk with petals. 66.4% of the nucleotides are present in helical regions.

It should be stressed that although some parts, like the longer hairpins, are firmly supported by experimental evidence, other aspects of the model should be regarded as a preliminary working hypothesis. Indeed, many choices were only based on the stability constants, defined by TINOCO *et al.* (1971). Undoubtedly, and as these authors also pointed out, the theoretical evaluation of the stability of polynucleotide conformations will certainly be refined in the future. Recently, CROTHERS and collaborators (DELISI and CROTHERS, 1971; CRALLA and CROTHERS, 1972) have already proposed improved estimates in this sense, and these will require a reevaluation of some parts of the model. We believe, however, that these improvements will mainly involve details rather than the general outline of the proposed mode, as at least a large part is experimentally supported by several lines of evidence.

Undoubtedly, the "flower"-model, as shown in fig. 7, is further folded in an intricate, three-dimensional superstructure, which plays an important role in the biological expression of the viral functions.

B. Genetic Code

Most of the nucleotide sequences were originally recognized as part of the coat gene because they correspond to known amino acid sequences.

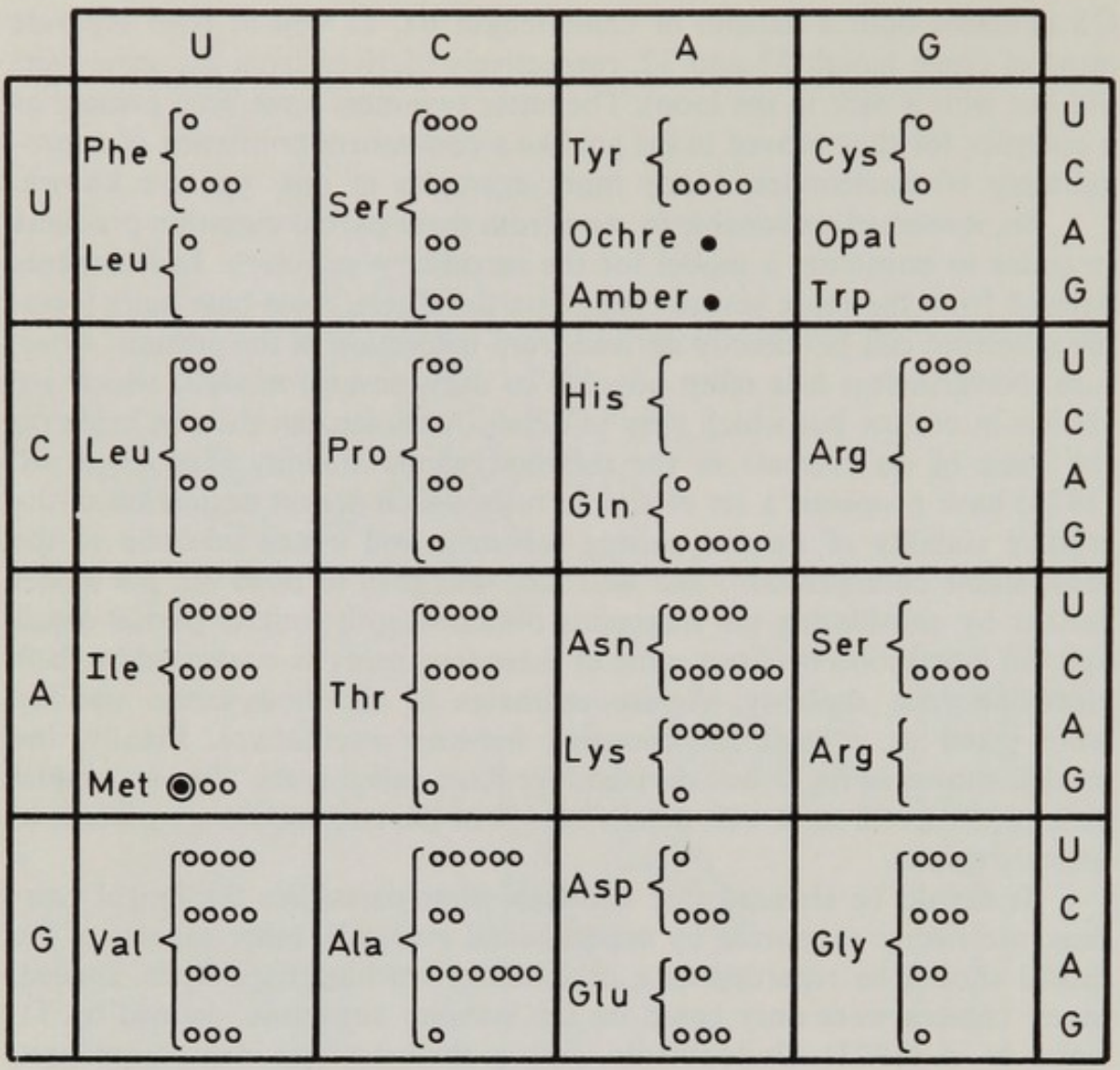

FIG. 8. Codons found in the MS2 coat protein gene.

When the complete sequence was available, however, it became clear that the amino acid sequence of the MS2 coat protein is not identical to the R17 sequence. In MS2 we found at position 11 Asp, at 12 Asn and at 17 Asp. All three differences involve Asp↔Asn changes which correspond to a single transition (A-A-Pyp↔G-A-Pyp). The correctness of this sequence has been independently confirmed by VAN DE KERCKHOVE and VAN MONTAGU by peptide analyses (personal communication). Can these exchanges explain the slight difference in immunological properties between MS2 and R17 (SCOTT, 1965; KRUEGER, 1969)?

As we have now established the complete nucleotide sequence of the coat gene as well as the correct sequence of the 129 amino acids of the coat polypeptide, we can derive directly the genetic code table (fig. 8). Not unexpectedly, the genetic code word dictionary compiled by OCHOA-NIRENBERG-KHORANA is fully confirmed. In all 49 different sense-codons

Table II. Differences found in the coat protein gene between MS2 and R17.

Amino acid position	MS2		R17		Position in codon
	Nucleotide	Nucleotide in opposite strand	Nucleotide	Nucleotide in opposite strand	
38	U	G	C	G	3
60	C	G	U	G	3
66	A	–	G	–	3 (s.s; loop)
69	C	G	U	A	3 (slippage)
90	C	A	U	A	1 (Leu codon)
91	C	–	U	–	3 (s.s.; loop)
98	U	G	C	G	3
127	C	G	U	G	3

are used. For a few amino acids the choice between the degenerate codons seems to be non-random, although this could still be a coincidence. We have already sequenced parts of the polymerase gene and of the A-protein gene from which we can again deduce a set of code words (CONTRERAS *et al.*, 1972, and unpublished results). In this way we found that in fact *all* 61 sense-codons are used in the phage message.

What dictates the choice between synonymous code words? It could constitute the basis of a modulation type of control, whereby in genes which have to be expressed at lower efficiency, particular codons brake the speed of the ribosomes, e.g. because the corresponding tRNA is present in low concentration or has a lesser affinity for it. Candidates for such rate-braking codons are A-U-A for isoleucine, A-C-A for threonine, U-A-U for tyrosine, A-G-U for serine and A-G-A and A-G-G for arginine. But many more data are needed to prove or disprove the modulation theory. Another possibility is that the choice between degenerate code words may be dictated by secondary structure requirements for the viral RNA. In fact the percent third letter nucleotides involved in base pairing is slightly higher than the average, but the data are too limited to be statistically significant. In this connection it is interesting to compare also the base changes between MS2 RNA and R17, for which five hairpins have been sequenced (SANGER, 1971). Eight base changes are found on a total of 218 nucleotides available for comparison (table II). All are transitions and 7 are C↔U changes. Largely because of the possibility of G—U base-pairing, 7 out of the 8 base changes do not interfere with the secondary structure. All codons sofar found in the R17 fragments are also used in the MS2 coat gene (i.e. the total number does not exceed 49).

In conclusion, it seems that often the choice between the degenerate codons is apparently non-random, but what dictates this choice is not clear: for some it could be modulation, for others it could be secondary and tertiary structural requirements or still other effects.

C. The Polarity Effect

It is known, both from *in vivo* and *in vitro* results, that at least part of the coat gene has to be translated in order to allow expression of the succeeding RNA polymerase cistron (LODISH and ZINDER, 1966; GUSSIN, 1966; ENGELHARDT *et al.*, 1967; ROBERTS and GUSSIN, 1967). Coat amber mutants at position 6 are strongly polar under non-permissive conditions, while similar mutants at positions 50, 54 or 70 are not polar. A logical explanation is that polarity is relieved when ribosomes travel over a region somewhere between positions 6 and 50 of the coat cistron.

This hypothesis can now be explained in molecular detail by the "flower"-model. Indeed, when ribosomes translate the region of amino acids 24 to 32 of the coat cistron, they have to open up the hairpin region c (fig. 7). In this way, they release the opposite strand, and, conceivably, this is sufficient for other ribosomes to bind at the initiating A-U-G codon of the RNA-polymerase gene.

D. The Specificity of Mutagenesis

If translations alone are legitimate, then amber codons (U-A-G) could arise either from the glutamine codon C-A-G or from the tryptophane codon U-G-G. A large series of amber mutants have been isolated for the phages f2, R17 and MS2 (table III). The location of the mutation in the coat cistron mutants was characterized by analysis of the polypeptide made under permissive conditions, e.g. in a su_I^+-strain where serine is

Table III. Amber-mutants in the coat gene.

Phage	Mutagen	Mutant	Mutation at position	Mutated codon
f2*	HNO_2	sus 3	6	C-A-G
	HNO_2	sus 11	70	C-A-G
R17†	HNO_2	B_2	6	C-A-G
	HNO_2 or FU	B_{23} to $_{29}$		
	HNO_2	B_{11}	50	C-A-G
	FU	B_{22}		
	HNO_2 or FU	B_{17} to $_{21}$	54	C-A-G
MS2††	HNO_2	908	6	C-A-G
	HNO_2	904	50	C-A-G
	NH_2OH	623 etc (5 mutants)		
	NH_2OH	601	70	C-A-G

*ZINDER and COOPER, 1964; WEBSTER *et al.*, 1967.
† GUSSIN, 1966; TOOZE and WEBER, 1967.
†† VAN MONTAGU, 1966; FIERS *et al.*, 1969; VANDEKERCKHOVE *et al.*, 1971.

introduced at the mutated site. All mutations, for the three related phages, occur only at position 6, 50, 54 or 70. It is of interest to examine these mutation data in the light of the structural model (fig. 7). Indeed, the C-A-G (Gln) codons at those positions where mutations can occur are all involved in a discontinuity of the double-stranded hairpin stem (an A or C-residue is looped out). The C-A-G (Gln) codon at position 40, where a mutation never has been found, forms part of an uninterrupted bihelical segment (either the mutagens do not attack this C, or else base-pairing in this region is critical and an induced second mutation in the opposite strand would give an unacceptable missense). The only other Gln-codon in the sequence, at position 109, is coded by a C-A-A codon, which would result in a non-viable ochre mutation (although in some of our stocks, a C-A-G variety at this position was also present). We cannot explain, however, why position 6 mutants are only obtained with nitrous acid as a mutagen and not with hydroxylamine, nor why position 54 mutants have sofar only been found for R17.

V. Conclusion

Due to the development of new methodology, great strides have been made in the determination of the primary structure of the viral RNAs. Many important regions of the phage MS2 RNA have been sequenced, and technically it now seems feasible to establish the complete structure within reasonable time. These results have offered a molecular basis for the understanding of several important biological phenomena and have opened new insights into the role of these viral RNAs. Nevertheless, many functions will have to be explained in terms of the 3-D structure of the RNA, and although some model building is at present possible, a clear understanding of the secondary and tertiary structure seems still a long way in the future.

VI. Acknowledgment

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THE DIVIDED GENOME OF THE BROMOVIRUSES

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CONTENTS

The divided genomes of the bromoviruses

I. Summary

The bromoviruses have 4 RNA components encapsulated into at least 3 classes of particles which can be distinguished by their buoyant densities. The heaviest particles contain an RNA of 1.1×10^6 daltons, the lightest an RNA of 1.0×10^6 daltons. The intermediate density particles contain jointly encapsulated RNAs of 0.75×10^6 and 0.3×10^6 daltons. The three largest RNAs are required for infectivity, and the third largest contains the coat protein gene. The smallest RNA also contains the coat protein gene, but not in a genetically transmissible form. Mutations in any of the three required RNA components can alter the physiology of virus infection. The way is open for characterization of the non-coat genes and for a detailed analysis of the coat protein gene.

II. Introduction

Over the past ten years the RNA bacteriophage system has proved highly successful for investigating the viral infection process. The genetic simplicity and flexibility of the host, the short generation time of the virus, and the simplicity of virus assay have allowed identification of the three viral genes and their products. We now have a reasonably sophisticated understanding of RNA bacteriophage replication and its associated control mechanisms.

The major thrust in virus research today is toward a similar understanding of the viruses which infect eucaryotic organisms. Animal virology is making rapid progress in this direction, but plant virology is beset with difficulties. Plant viruses have low specific infectivities. Monolayer assay systems are not available and assays require days to weeks. Plant viruses infect asynchronously, and during the early stages of growth only a small

fraction of the plant's cells are infected. The hosts are genetically ill-defined and are not amenable to rapid genetic analysis. Nonsense suppressors are unknown in plants, and until recently genetic recombination was unknown in plant viruses. The only saving grace of plant viruses has been their ease of isolation and consequent suitability for physical and chemical studies.

In recent years, striking differences between animal and bacterial viruses have become apparent. One of the major differences is associated with the initiation of protein synthesis. While bacterial viruses can independently initiate synthesis of several proteins from a single RNA strand, the animal viruses can initiate only one polypeptide per RNA strand. Different animal viruses have circumvented this difficulty in different ways. In poliovirus the single polypeptide is split by enzymes into the multiplicity of proteins that the virus requires during infection. Other viruses such as influenza and reovirus contain multiple RNA components, each of which appears to contain a single gene.

There is reason to suspect that restriction to a single initiation point per RNA chain may be a general feature of eucaryotes. Indeed, the existence of a unique class of viruses, the divided genome plant viruses, might be taken as evidence for this. The divided genome plant viruses have multiple RNA components, as do many animal viruses, but the RNA components are independently encapsulated to give a virus which requires two or three particles to produce infection. In contrast to the more complex animal viruses which contain negative (untranslatable) strands or double-stranded RNA, the divided genome viruses contain plus strands, and free viral RNA is fully infectious. Individual RNA components can be separated, manipulated and recombined at will, greatly facilitating genetic analysis.

The divided genome phenomenon, although apparently confined to plant viruses, is by no means uncommon. Of the 16 groups of plant viruses recently designated by HARRISON *et al.* (1971), seven clearly have divided genomes.

III. The Bromoviruses

The bromoviruses are the best characterized of the divided genome viruses. The name "bromovirus" derives from brome mosaic virus (BMV) which is the prototype for this group. Cowpea chlorotic mottle virus (CCMV) and broad bean mottle virus (BBMV) are also included in this group because of their similar physical properties. The bromoviruses have icosahedral symmetry (fig. 1), molecular weights of about 5 million daltons, RNA contents of about one million daltons, and contain a single protein of about 20,000 daltons. The viruses are serologically distinct,

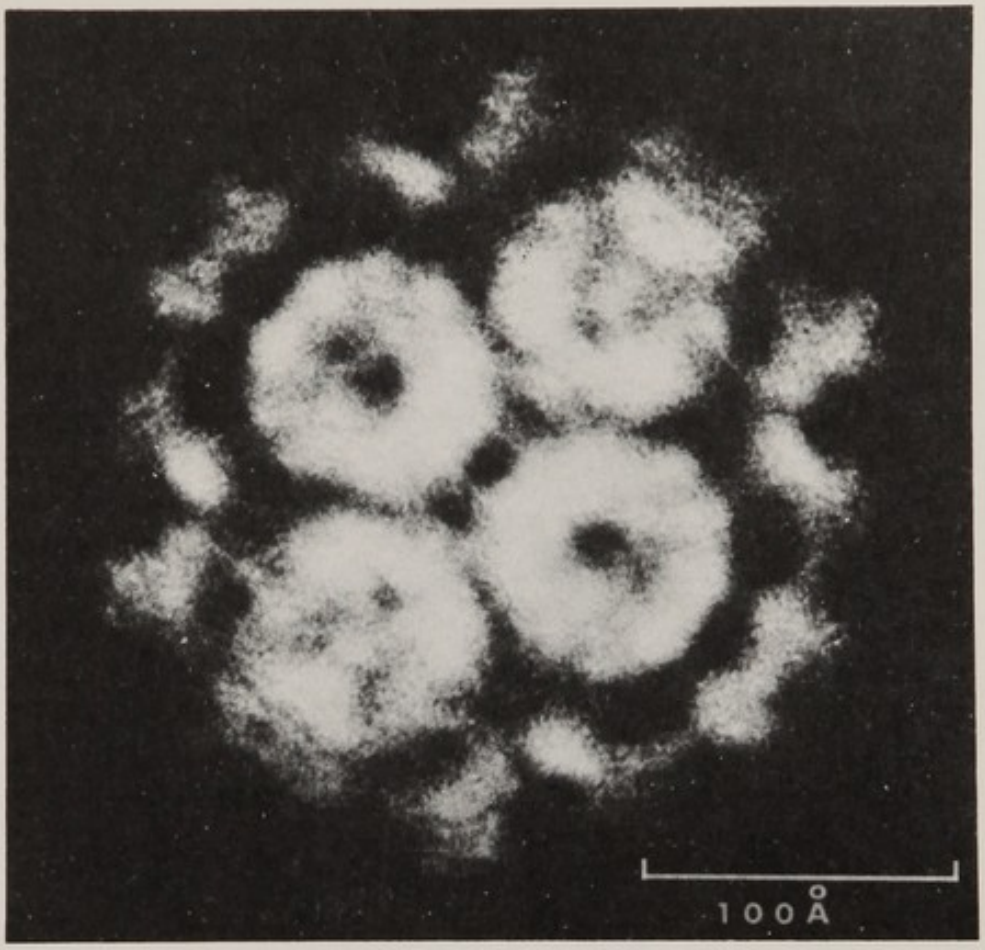

FIG. 1. An "average" picture of CCMV, oriented precisely in the two-fold position. The picture was obtained by superimposing four views of the particle rotated 180° with the negative unreversed and reversed. There are 32 morphological units, 12 composed of 5 structure units each (pentamers) clustered about five-fold axes and 20 composed of 6 structure units each (hexamers) clustered about three-fold axes. The strain is 1% uranyl acetate, pH 4.7 (BANCROFT, 1970).

although a distant relationship has recently been observed between BMV and CCMV (SCOTT and SLACK, 1971). The host ranges of the viruses are markedly different though they all produce local lesions on several members of the Chenopodiaceae (see fig. 2). The bromoviruses are somewhat unusual in being most stable in acidic solutions (pH 3-6). The individual bromoviruses differ in surface charge. BMV is positively charged throughout the range of highest stability. CCMV is a more "typical" acidic virus while BBMV has an intermediate isoelectric point.

Initially, the bromoviruses were thought to be among the simplest viruses, and only recently has their distinction from the RNA bacteriophages become clear. It is now apparent that each of these viruses has 4 RNA components (fig. 3), which have been numbered for convenience one to four in order of decreasing size. The RNA components have molecular weights of roughly 1.1, 1.0, 0.8 and 0.3 million daltons although gel electrophoretic comparison of denatured viral and *Escherichia coli*

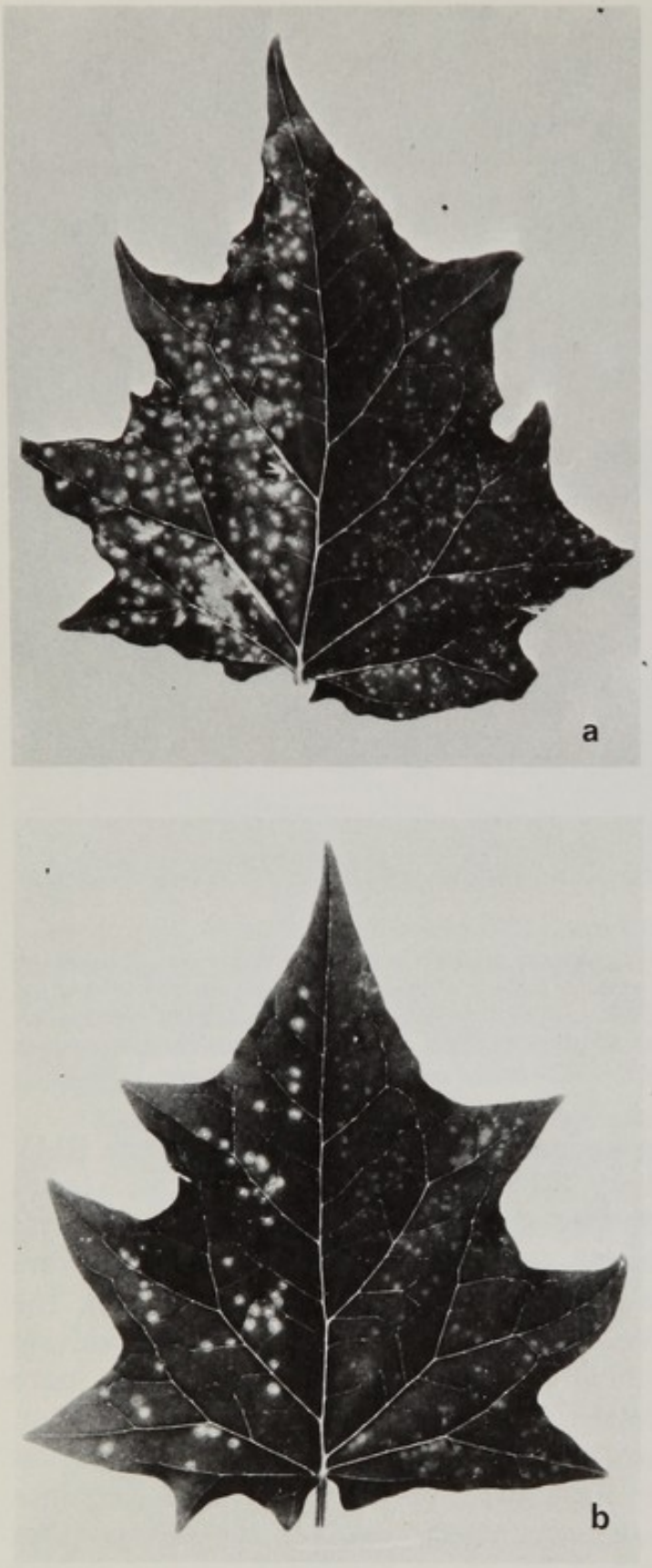

FIG. 2. Local lesions produced by BMV strains on *Chenopodium hybridum*. *(a)* wild-type (left) and a small lesion variant (right). *(b)* wild-type (left) and BMV-F (right) (BANCROFT and LANE, in press).

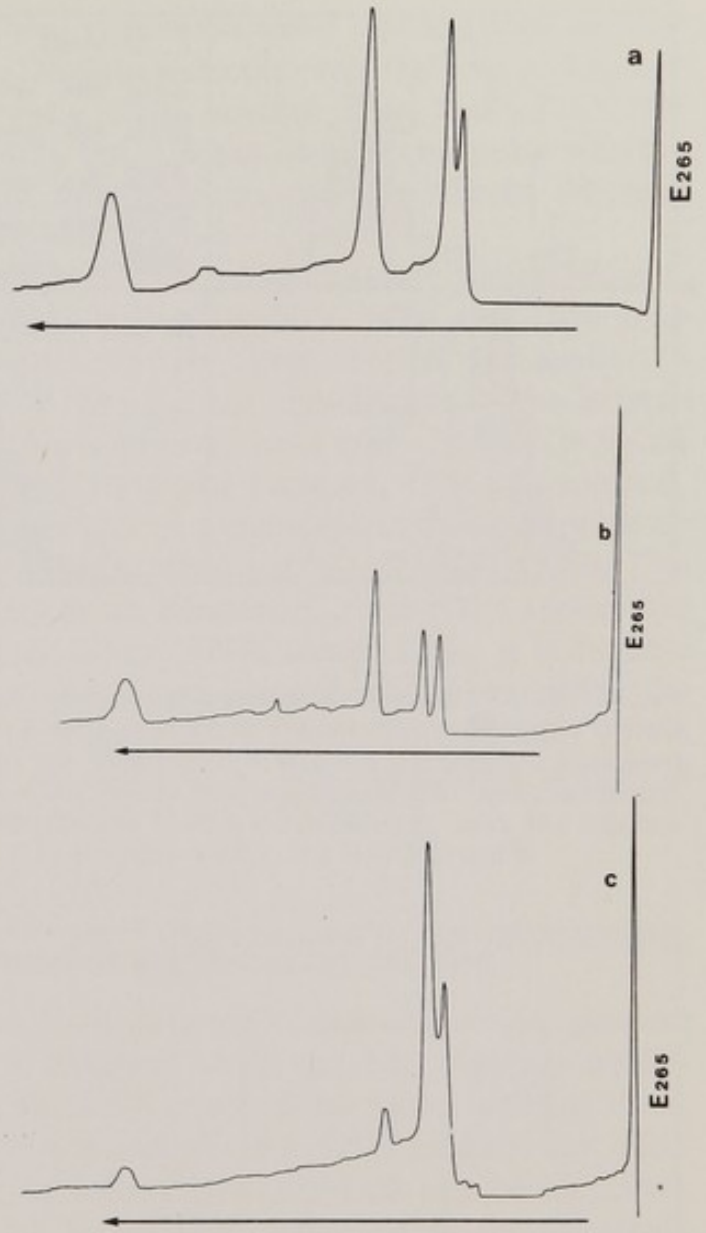

FIG. 3. Gel electrophoretic separations of the RNAs of the bromoviruses: *(a)* BMV; *(b)* CCMV; *(c)* BBMV. The RNAs have been modified with formaldehyde by a method similar to that of BOEDTKER (1971) and electrophoresed on 2.5% polyacrylamide gels (without formaldehyde) according to the procedure of LANE and KAESBERG (1971). Small amounts of RNA (less than two micrograms) were applied to the gels, which were scanned at 265 nm after staining with toluidine blue 0. Absolute mobilities are not comparable.

ribosomal RNAs suggests that they may be larger than previously supposed (1.3, 1.2, 0.85 and 0.3 million daltons).

The two larger RNAs are encapsulated into particles with distinctly different buoyant densities. BBMV has only a small amount of RNA component 3 (fig. 3), and the compositions of the particles containing the two smaller RNAs are uncertain. In BMV and CCMV the two smaller RNAs are contained in a class of virions with intermediate density (figs. 4 and 5).

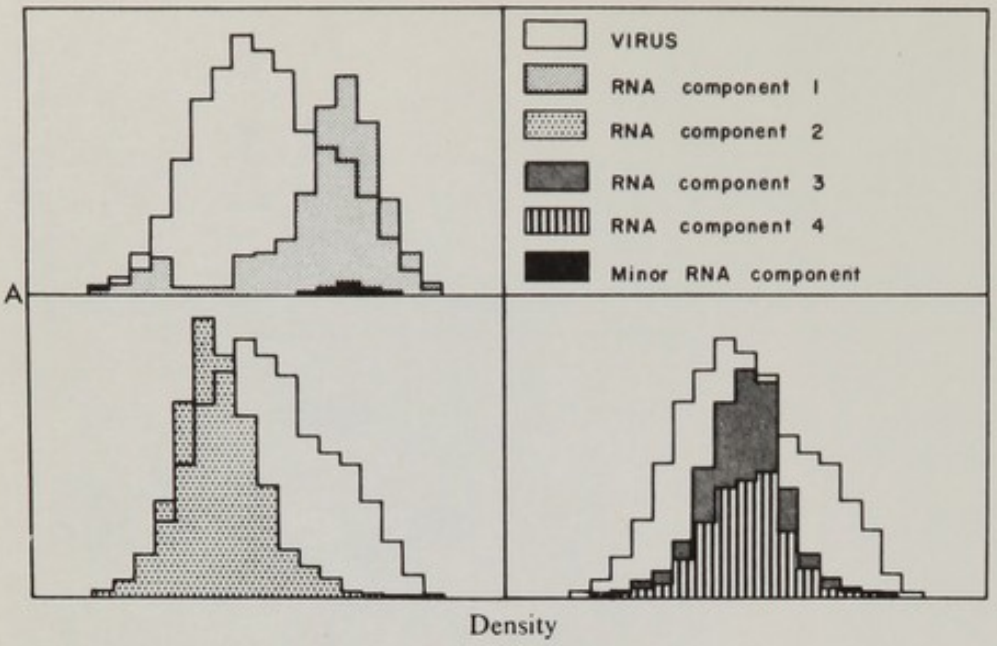

FIG. 4. Distribution of RNA species after equilibrium centrifugation of BMV in 37.5% RbC1. Optical densities were determined on fractions from a preparative gradient; RNA was isolated from each fraction, and component composition was determined by gel electrophoresis. RNA distributions have been divided into three graphs, and a tracing of the distribution of intact virus included with each. The absorbance scale of virus distributions has been compressed by a factor of two relative to that of RNA distributions (LANE and KAESBERG, 1971).

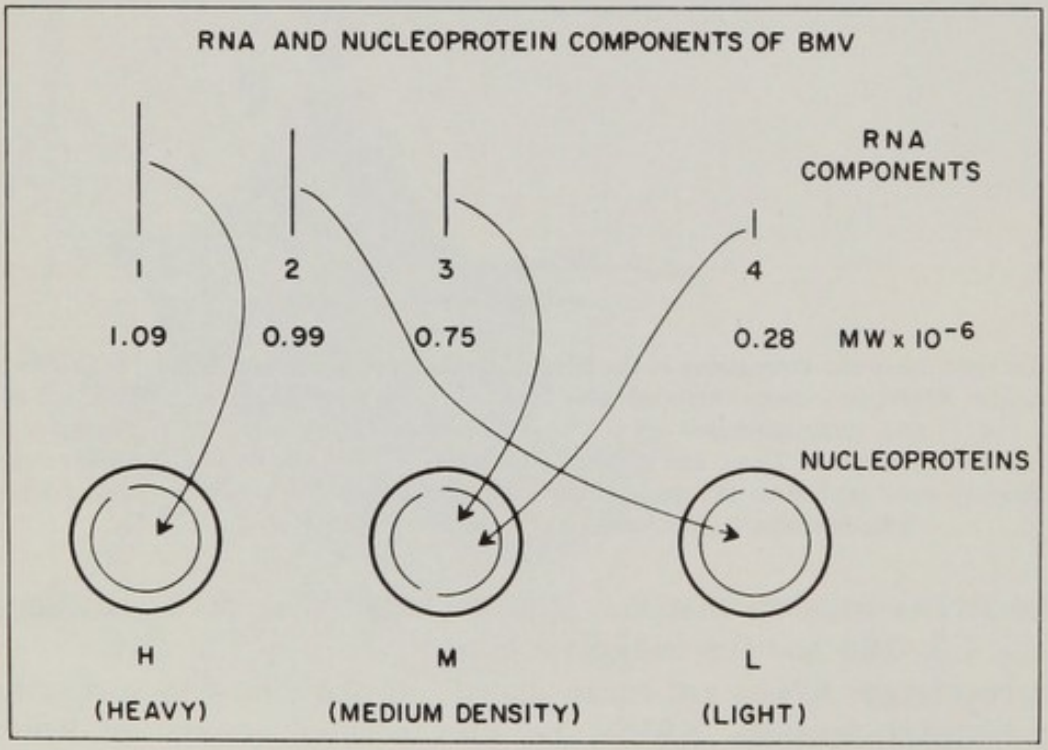

FIG. 5. Relationships between the RNA and nucleoprotein components of BMV. The RNA components are schematically represented with size decreasing from left to right, and the nucleoproteins are represented with density decreasing from left to right (LANE and KAESBERG, 1971).

The coat proteins of the three viruses are easily distinguished by their amino acid compositions. By SDS gel electrophoresis the coat proteins of BMV and BBMV are apparently 20% smaller than that of CCMV (AGRAWAL and TREMAINE, 1972), but it is not clear at this point whether this represents an actual size difference or merely reflects differing behavior of the proteins in detergent solution.

The end groups of the BMV RNA components are similar. The 5' termini are complex; 40% pAp, 25% pUp, 20% pGp and 15% pCp (FRAENKEL-CONRAT and FOWLKS, 1972). These termini are similar to those of tobacco mosaic virus RNA, but the significance of the heterogeneity is unclear. All four RNA components have pGp-Cp-Cp-Cp-A-OH at the 3' terminus (LEPPLA, 1970; GLITZ and EICHLER, 1971). In addition, all of the BMV RNA components can be aminoacylated with tyrosine by a mixture of plant aminoacyl tRNA synthetases (HALL *et al.*, 1972). This implies quite extensive homology at the 3' termini. Since RNA replication is presumably similar for all the BMV RNA components, it is feasible that the tRNA-like structure at the 3' end is involved in recognizing the RNA replicase. The protein synthesis chain elongation factor "T_s" is known to bind aminoacyl tRNAs and also to be a constituent of the $Q\beta$ bacteriophage replicase (BLUMENTHAL *et al.*, 1972).

IV. The Genetics of the Bromoviruses

By separating components from different strains of a divided genome virus and recombining them in different ways, one can associate phenotypic differences with the specific RNA components that determine them. The greater the number of RNA components a virus requires for infectivity, the greater the number of linkage groups which can be identified. Since the bromoviruses require three RNA components for infectivity (the maximum known at this time), they are among the most favorable plant viruses for genetic analysis.

The genetic complexity of the bromoviruses was not recognized in the earlier stages of their characterization. Before the advent of gel electrophoresis the two smaller components of BMV RNA were considered the product of a single phosphodiester bond cleavage of the "large component". However, the RNA components can be sufficiently separated by gel electrophoresis to show that mixtures are more infectious than isolated components. With both BMV and CCMV it is clear that omitting any of the three largest RNA components from the inoculum greatly decreases the specific infectivity (table I; LANE and KAESBERG, 1971; BANCROFT and FLACK, 1972). The obvious conclusion is that the three largest RNAs are all required for infectivity. BBMV is more difficult to assess. Each of the two largest RNAs has at least two configurations,

and the larger RNA in the compact configuration is not easily separated from the smaller in the extended configuration. The third largest RNA of BBMV is present in extremely small amounts (fig. 3). It is clear, however, that at least the two largest RNAs are required for infectivity (HULL, 1972).

Having separated the three biologically active components, one can

Table I. Infectivity of mixtures of RNA components.

Leaf No.	1	2	3	4	5	6	1-6
Lesions (RNA 1+2)	0	0	0	0	0	0	0
Lesions (RNA 1+2+3)	30	32	17	48	48	38	213
Leaf No.	1	2	3	4	5	6	1-6
Lesions (RNA 1+3)	2	2	5	1	2	3	15
Lesions (RNA 1+2+3)	38	47	125	18	13	63	304
Leaf No.	1	2	3	4	5	6	1-6
Lesions (RNA 2+3)	1	5	9	3	10	6	34
Lesions (RNA 1+2+3)	64	172	189	48	160	76	706

Assays were performed on half leaves of *Chenopodium hybridum* which were dusted with 600 mesh carborundum; each preparation was inoculated on equal numbers of left and right half leaves. Samples were diluted to 2 μg/ml, equally divided among the components, with inoculation buffer (10 mM sodium acetate, 1 mM magnesium acetate, adjusted to pH 5.5 with acetic acid) plus 0.5 mg/ml bentonite. RNA components were isolated from gels after electrophoresis by soaking gels for 10 sec. in inoculation buffer plus 0.05% toludine blue 0, and then soaking for 2 h in the buffer at 0° C in the dark. Partially stained bands were excised with a razor blade and homogenized in inoculation buffer plus 0.5 mg/ml bentonite. Concentrations of RNA components were calculated from the total amount of RNA applied to the gels and the component proportions as estimated from scans similar to that shown in fig. 2 (LANE and KAESBERG, 1971).

Table II. The six possible hybrids (A-F) that can be constructed between two strains of a three component virus. "W" represents a wild-type RNA species, and "V" represents a variant RNA species.

RNA Species	HYBRIDS					
	A	B	C	D	E	F
1	W	W	V	V	V	W
2	W	V	W	V	W	V
3	V	W	W	W	V	V

then construct genetic hybrids. Six different hybrids can be constructed from a pair of three component viruses (table II). For genetic analysis one needs only to obtain phenotypically differing strains of the viruses. The uniformity of natural strains of the bromoviruses poses an obstacle to genetic analysis. The first analyses were performed using naturally occurring mutants of BMV which were selected for their abnormal electrophoretic mobilities (LANE and KAESBERG, 1971). When genetic hybrids were constructed, the electrophoretic mobility (and therefore the coat protein) was found to be determined by RNA component 3 (fig. 6).

Since the different members of the bromovirus group have widely diverging properties, construction of genetic hybrids among the members of the group would facilitate genetic analysis. However a general rule with divided genome viruses is that distant serological relatives are genetically

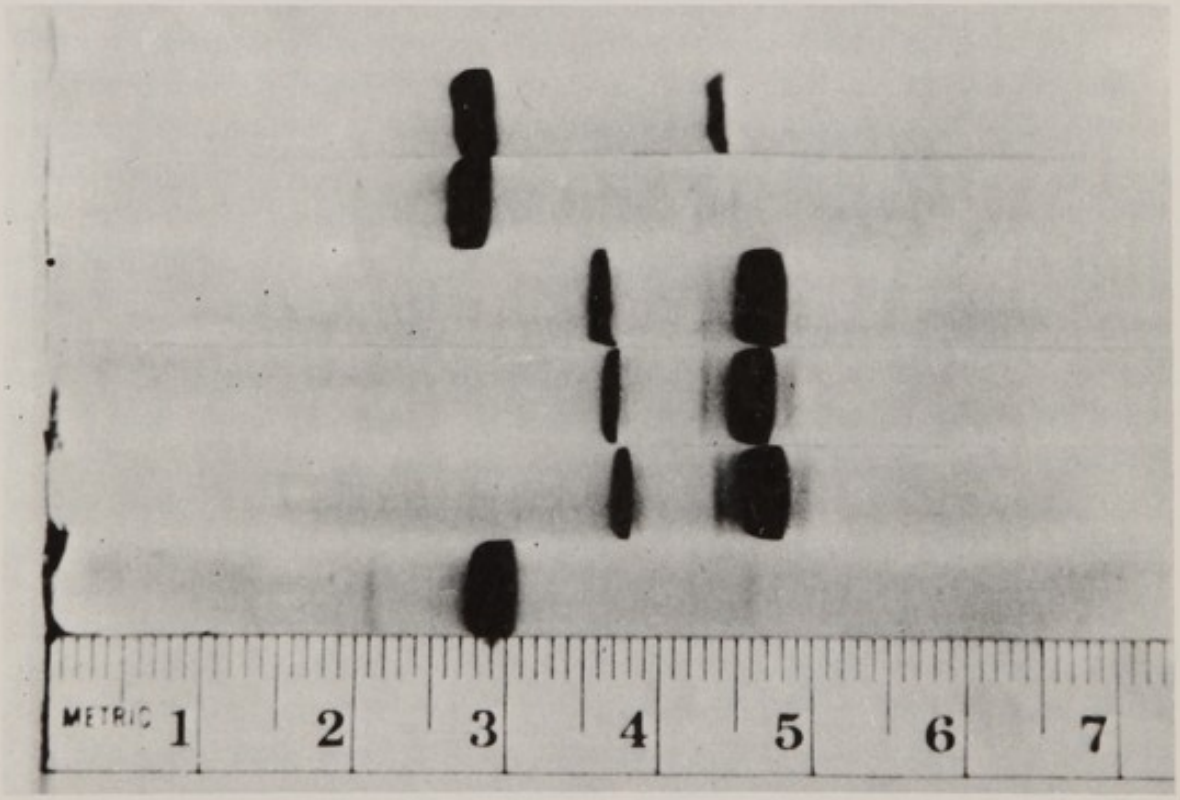

FIG. 6. Gel electrophoresis of hybrids of wild-type and an electrophoretic variant of BMV. From left to right are hybrids a-f of Table II. Slowly migrating bands (dimers) are prominent in hybrids with wild-type mobility (LANE and KAESBERG, 1971).

incompatible. It has, however, been possible to construct a hybrid which has BMV RNA species one and two and CCMV RNA species three (BANCROFT, 1972). The hybrid is serologically identical to CCMV. It grows very poorly on the local lesion host, producing only minute lesions. It has so far proved impossible to grow the hybrid on hosts normally infected systemically by either BMV or CCMV, even using high inoculum

concentrations and very sensitive assays for virus growth. The specific infectivity of the hybrid is low, and this may reflect the fact that it contains no detectable RNA species 3.

The existence of the genetic hybrid is an additional indication of the relationship between BMV and CCMV. The poor growth of the hybrid reflects the genetic incompatibility of the two parental viruses. The mechanism for this incompatibility is intriguing, but the poor growth of the hybrid precludes the study of its metabolism. Attempts to produce additional genetic hybrids between BMV and CCMV have so far been unsuccessful. No attempts have been made to construct "intervirus" genetic hybrids using BBMV.

V. The Smallest RNA Component

The smallest RNA component is not required for infectivity by any of the bromoviruses. The small RNA species of BMV has been studied in some detail. SHIH *et al.* (1972) have shown that it contains 950 nucleotides based on the yields of selected oligonucleotides following pancreatic ribonuclease digestion (fig. 7). The agreement between this number and the gel electrophoretic molecular weight indicates that the small RNA is a single component. Comparison of the large pancreatic ribonuclease oligonucleotides among the BMV RNA components shows that RNA species 4 contains a subset of the sequences from RNA species 3. The close similarity of sequences shows beyond reasonable doubt that RNA species 4 must be derived from RNA species 3.

Recently SHIH and KAESBERG (in press) have found that RNA species 4 directs synthesis of coat protein in a cell-free protein synthesizing system derived from wheat embryos. This confirms that RNA species 4 is derived from species 3 and shows that it too contains the coat protein gene. Attempts to influence virus coat protein genetics by adding excess RNA species 4 of wild-type virus to inoculum containing a mixture of species 1, 2 and 3 of an electrophoretic variant have been unsuccessful. RNA species 4 therefore cannot contribute genetic information to the infection and must be derived from species 3 during infection.

Preliminary studies indicate the existence in virus-infected barley of an RNA replicative form corresponding to species 4. Thus it is likely that species 4 replicates independently in the cell after the initial event by which it is derived from species 3. The production of RNA species 4 may be a manifestation of a mechanism for overcoming the single initiation point limitation of eucaryotes. This hypothetical mechanism allows the virus to produce large amounts of coat protein and at the same time to avoid excess production of non-coat proteins which are needed only in catalytic amounts.

Three simple genetic tests can be applied to divided genome plant viruses. Formation of genetic hybrids is the most stringent of these. To be confident of the results one must do careful controls to assure the biological purity of the isolated RNA species from which the hybrids are constructed. This is simple for two component viruses where low specific infectivity is a satisfactory criterion, but for three component viruses one

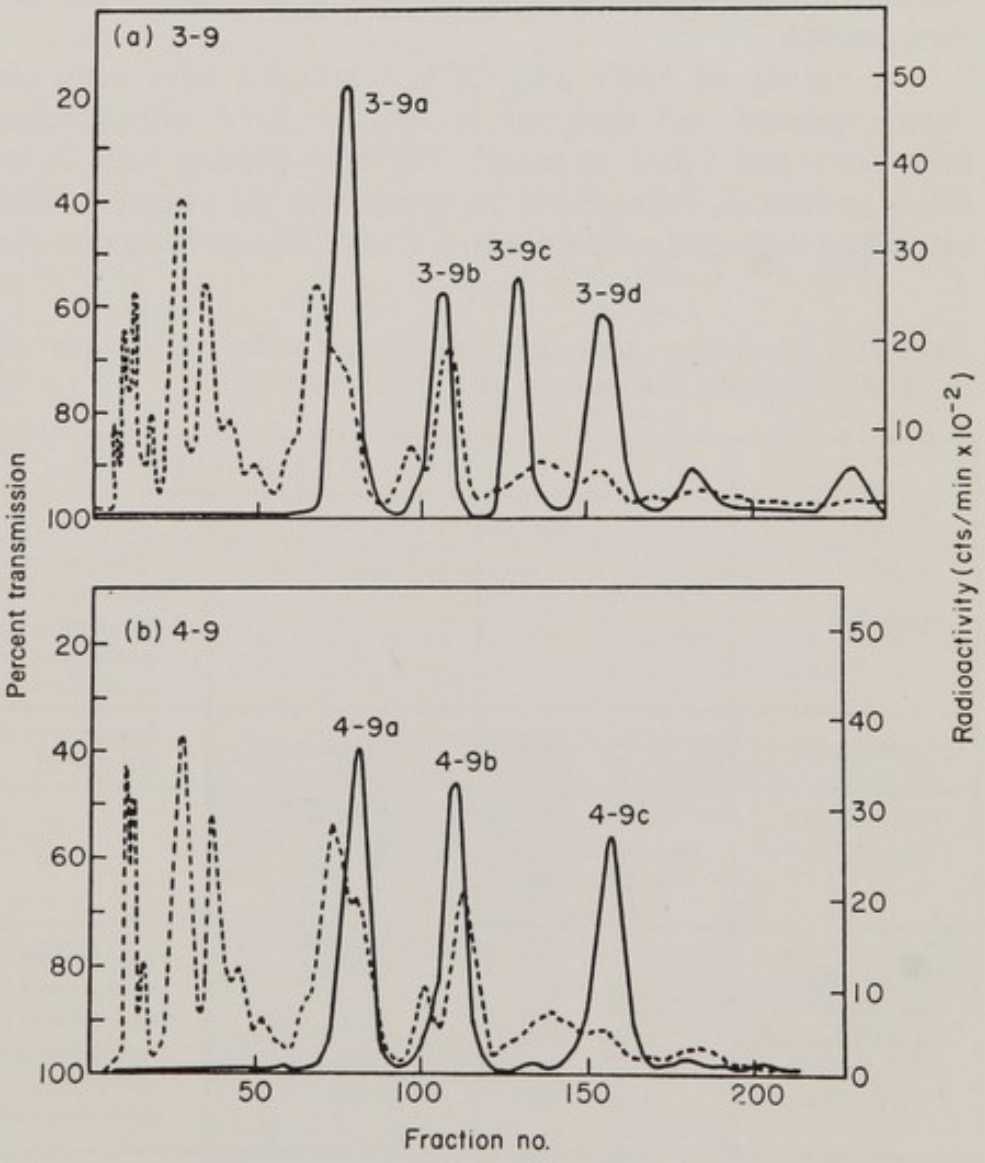

FIG. 7. DEAE-Sephadex rechromatography of the nonanucleotides derived from components three and four of BMV RNA. Oligonucleotides were eluted with a linear gradient of zero to 0.45 M NaCl containing 7 M urea adjusted to pH 3.1 with HCl.

(a) Nonanucleotides from component three. *(b)* Nonanucleotides from component four. Solid line = radioactivity; broken line = percent transmission of an internal standard of a complete pancreatic ribonuclease digest of unfractionated BMV RNA (SHIH *et al.*, 1972).

must test appropriate mixtures of components since a highly contaminated species may still exhibit low specific infectivity (BANCROFT and LANE, in press).

For small lesion mutants, which grow poorly, a "supplementation test" is valuable. This involves producing an inoculum by mixing a purified wild-type RNA component with an extract of mutant-infected tissue. The mutated RNA species is identified by determining which of the wild-type species causes the mixture to produce wild-type lesions.

The third test is the complementation test, which involves inoculating mixtures of lesion type mutants to see if they give rise to wild-type lesions. Normal lesions appear only where the mutations are on different RNA components.

A variety of BMV and CCMV mutants have been classified into linkage groups and assigned to specific RNA components (table III; BANCROFT and LANE in press). The coat protein gene is invariably on RNA species 3. Alterations in symptoms on systemic hosts are often associated with coat mutations in CCMV. The co-reversion of coat protein

Table III. Phenotypes associated with different RNA species from various mutants of CCMV and BMV (BRANCROFT and LANE, in press).

Mutant	RNA component		
	1	2	3
CCMV(ts)[1]	–	–	symptoms on systemic host RNA component ratio coat protein specific infectivity temperature sensitivity
CCMV(mild)[2]	–	local lesions[3]	symptoms on systemic hosts coat protein
CCMV(MC2a) CCMV(MC2d)	– temperature-sensitivity of lesion production	local lesions local lesions	– –
BMV(MB1b) BMV(MB2a) BMV(MB4a) BMV(MB4b) BMV(EV2b and 5)	– – – – –	– – – – –	local primary lesions local primary lesions local primary lesions local primary lesions coat protein RNA component ratio
BMV(F)	–	local primary lesions[4] RNA component ratio	–

[1] See BANCROFT *et al.*, 1972.
[2] See LANE and KAESBERG, 1971.
[3] Local lesions on *C. hybridum*.
[4] Local lesions on *C. hybridum* and primary lesions on *C. quinoa*.

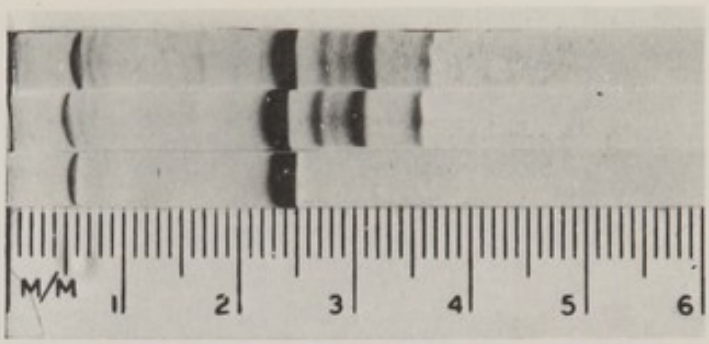

FIG. 8. Gel electrophoresis of BMV strains. Left to right are wild-type and two different preparations of BMV-F. Electrophoretic conditions are described by LANE and KAESBERG (1971).

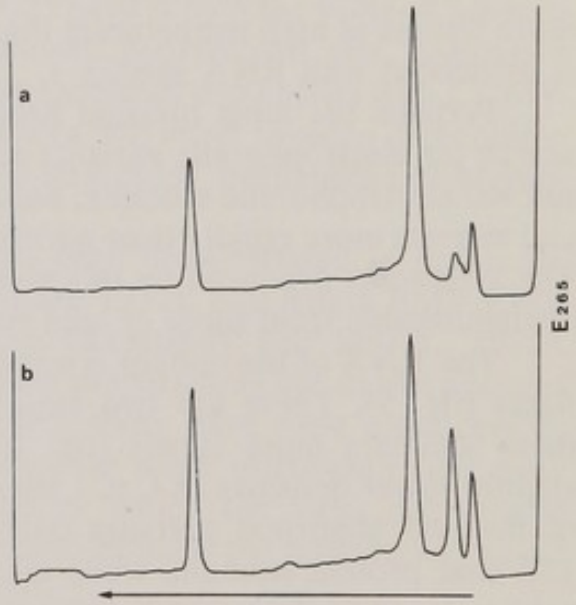

FIG. 9. Gel electrophoresis of RNA of BMV strains. a) BMV F, b) wild-type. Electrophoresis is on 3.2% polyacrylamide gels under conditions described by LANE and KAESBERG (1971).

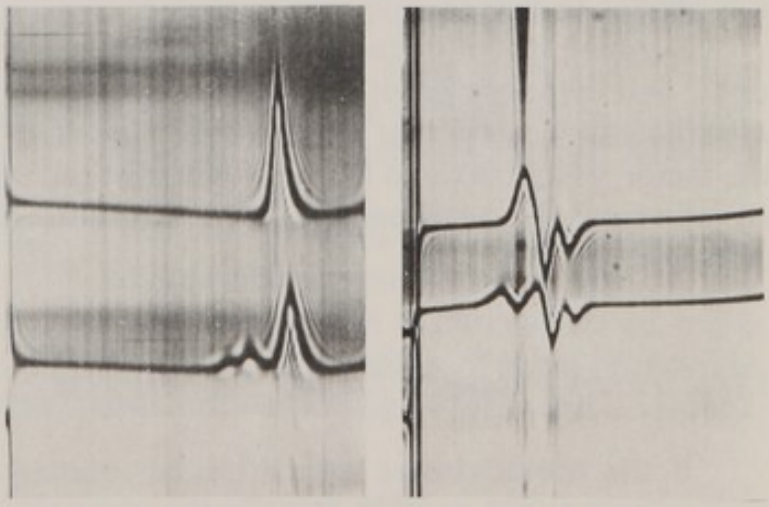

FIG. 10. Sedimentation velocity patterns of BMV strains. Upper is wild-type; lower is BMV-F.

FIG. 11. Sedimentation equilibrium patterns of BMV strains in CsCl. Upper is wild-type; lower is BMV-F.

type and symptom type in one such mutant suggests that both may result from a single mutation. In BMV, changes of RNA component ratio are often associated with naturally occurring electrophoretic variants. Again there is good cause to believe that both changes result from a single mutation.

Small lesion phenotype in BMV associate with RNA species 3. So far, all known coat protein mutants have shown normal lesion morphology, and it is unclear if lesion morphology can be influenced by the coat protein gene. In CCMV the small lesion phenotypes associate with RNA species 2.

With CCMV one mutant has been obtained which produces many more lesions at high temperature than at low temperature. This phenotype is associated with RNA species 1.

Perhaps the most unusual bromovirus mutant was obtained in the search for electrophoretic variants of BMV. This mutant (BMV-F) has the normal electrophoretic mobility, but about 10% of its particles are smaller and migrate more rapidly than normal BMV on gel electrophoresis (fig. 8). The symptoms produced by this mutant on a local lesion host are clearly distinguishable from those of wild type virus (fig. 2b).

The RNA of the mutant is enriched in the two smaller RNA components (fig. 9). There are two major classes of small particles; both of these sediment more slowly (fig. 10) than wild type virus and band at slightly lower densities in CsCl than wild type virus (fig. 11). The larger of the two abnormal particles contains RNA species 3 and has about 150 coat protein subunits while the smaller has two molecules of RNA species 4 and contains about 120 coat protein subunits. The small particles have slightly smaller diameters in the electron microscope. They exhibit less structure than the wild type particles; but, where structure is apparent, it is similar to that of wild type particles.

Surprisingly, this unusual phenotype is inherited through RNA species 2 and therefore is not the result of an altered coat protein. Preliminary experiments suggest that the altered ratio of RNA components may be the factor which leads to the production of the small particles. Neither the structures of the small particles nor their mode of assembly is apparent at this time.

VII. Future Prospects for the Bromoviruses

If the bromoviruses infected either animal or bacterial cells, future experiments could quickly identify the virus-specific proteins and answer many of our questions about viral metabolism. However, since plant viruses possess all the unfortunate disadvantages discussed in the intro-

 duction, the course of future research will be greatly influenced by the

development of new techniques. Several approaches seem promising.

A detailed study of the genetic code in plants is feasible utilizing the bromoviruses and minor modifications of available techniques. Nucleotide sequencing of the smallest RNA components of the bromoviruses is almost within the grasp of present technology. The amino acid sequences of the bromovirus coat proteins can be obtained by well established techniques. Combining the two sequences will establish the genetic code in plants and allow its comparison to the code in bacteria. More detailed studies of coding and the control systems of translation will be possible utilizing the wheat embryo system.

Recently SAKAI and TAKEBE (1972) have shown the value of tobacco protoplasts for studying virus metabolism. MOTOYOSHI and BANCROFT (unpublished) have succeeded in infecting tobacco protoplasts with CCMV. It is also likely that the other bromoviruses will infect tobacco protoplasts. The way is therefore open to study the molecular mechanisms of bromovirus synthesis.

Virus-specific RNA synthesis has been demonstrated in plants infected with BMV and BBMV (cf. ROMERO, 1972). Full length BMV RNA species have been synthesized *in vitro* (KUMMERT and SEMAL, 1972). Unfortunately the viral RNA polymerase preparations are independent of added template. Attempts to purify the activity have been unsuccessful. Continued efforts to purify the polymerase seem worthwhile, not only because of interest in the mechanism by which the virus coordinates synthesis of its RNA components, but also because the polymerase would be a useful tool for sequencing the RNA.

By isolating additional mutants of the bromoviruses we hope first of all to get a feeling for the potential of plant viruses to show phenotypic variation. Secondly, we would like to know which RNA components influence particular phenotypes, and eventually we hope to learn how individual mutations alter the phenotypes. The genetic diversity of the plant kingdom is well worth exploring in studies of plant virus mutants. The discovery of a nonsense suppressor, for example, would have far reaching consequences for plant virology.

Our view of the biology of the bromoviruses is very different today than it was three years ago. If comparable progress can be made in the next three years we can expect research on the bromoviruses to make useful and unique contribution to our understanding of virus replication just as they have contributed to our understanding of virus self-assembly.

VIII. Acknowledgments

I would like to thank Drs. JOHN BANCROFT and ROGER HULL for helpful discussions and the American Cancer Society (Grant No. PF 756) for financial support.

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HIGH VOLTAGE LOW-TEMPERATURE ELECTRON MICROSCOPE STUDIES OF VIRUS STRUCTURE

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High voltage low-temperature electron microscope studies of virus structure

I. Introduction

Biologists and biomedical researchers agree that the elucidation of the molecular principles underlying structural and functional properties of membranes is a cardinal problem of modern biology, particularly since many major activities of normal and abnormal living cells involve membrane-associated reactions (1). Results of our high voltage cryo-electron microscope studies and research on ferritin-conjugated antibodies and ferritin-conjugated plant agglutinins, together with related investigations, have led a growing number of scientists to view membrane surfaces as mosaic composed of patches of lipid, areas of protein, and carbohydrate sites where the sugar end of a glycoprotein sits on the outer membrane (2-4).

Advances in instrumentation and preparation techniques correlated with biochemical studies have led to a better understanding of major virus constituents and their relation to host cell membranes at the molecular level. The high resolving power of the electron microscope is inherently capable of directly visualizing structures of molecular and pauci-atomic dimensions. Yet, before this unique potential can be successfully applied to the study of labile biological systems in their native state, we must implement new approaches in instrumentation and preparation techniques which would enable us to overcome the severe limitations imposed by specimen radiation damage, dehydration, thermal noise and electron optical aberrations.

Systematic efforts of electron microscopists, theoretical physicists and engineers in recent decades have brought us closer to this goal. Our own development of a modified high voltage microscope with improved

point cathode sources for controlled microbeam illumination, superconducting lenses and related instrumentation has been cited by many researchers as a major contribution to this field. As a result of these combined efforts, we have installed and routinely operated the first functioning prototype for a large-scale cryo-electron microscope system which enables us to consistently achieve 8Å to 16Å resolutions in specimens of cock retinal rod, nerve myelin, bacteriophages and related lamellar systems at temperatures of 1.8° to 4.2°K under conditions approaching the native hydrated state (9, 10, 13-20).

II. Developmental Background

A. Preparation Techniques and Early Work

When I first began my work in the field, biological specimens were prepared mainly by dehydration and embedding methods which deprived them of their inherent hydrated components. It also was impossible to carry out correlated studies, such as biochemistry, which required large amounts of untreated specimens.

The development of a modified microtome and related freeze-sectioning methods made it possible to obtain well-preserved ultrathin frozen sections of unembedded fixed or fresh material. My conception and development of the diamond knife and associated special microtome during the course of work at the Nobel Institute of Physics (5) made this approach even more practical. In sections ca. 100Å to 400Å thick, three-dimensional structure of biological tissues was preserved and we were able to obtain results which could be correlated with polarized light and phase contrast investigations.

Cryofixation and ultra-rapid freezing with liquid helium II were among the low temperature preparation techniques (6, 7) I used in the late 1950's (8), in a systematic study of serially arrested states of activity in biological systems and ice-crystal formation. The resulting data indicated that development of the unique potential inherent in the low temperature domain could well prove a key factor in advancing the study of life processes under conditions of minimum perturbation.

On the basis of these studies, it was possible to speculate that cryobiology might reveal new phenomena relating to the cumulative effect often associated with the high degree of molecular order characteristic of the solid state induced in biological specimens at temperatures close to absolute zero. It also became feasible to explore the relationship of low temperatures and specimen radiation damage.

B. Improved Point Cathode Sources and Superconducting Lenses

We have consistently used improved point cathode sources to provide stable, coherent microbeam illumination of high brightness, small spot size and low energy spread in the course of our work (9, 10).

BOX'S and BROIDA'S work (11, 12) on trapping free radicals and stabilizing ions at liquid helium temperatures further underscored the importance of the low temperature approach as an important tool in biomedical and biological research.

In developmental studies using superconducting lenses of 27,220 ampere-turns operating in the persistent current mode, resolutions of 10Å to 20Å were achieved during longer exposure times at lower beam intensities, thus reducing specimen radiation damage (13-15).

Since that time, the use of liquid helium temperatures in the objective system has permitted us to extend this work. Characteristic electron optical phenomena associated with trapped fluxes in thin superconducting films have been observed. In addition, a temperature-dependent anomalous electron transparency effect was demonstrated in 200 kV studies of 1000Å-thick lead films (14, 16). This may represent a whole range of new phenomena which can be visualized directly only at temperature in the liquid helium range.

C. Integrated High Voltage Cryo-Electron Microscope

Pursuing this approach, my colleagues and I have installed the large-scale Collins closed-cycle superfluid helium refrigeration unit, conceived of and built by Prof. SAMUEL C. COLLINS, in our laboratory. The 20-watt capacity unit is composed of over 36 feet of transfer lines and a novel heat exchanger, and it is installed in parts of a 5-story building.

This closed-cycle superfluid helium refrigerator has been fully integrated with the modified high voltage (200 kV) electron microscope and routinely used in over 165 successful experiments to significantly reduce specimen radiation damage, contamination and thermal noise during prolonged vibration-free examination of specimens at 1.8° to 4.2°K (17). At the same time, it enables us to combine these advantages with high penetration power, ultra-high vacuums, decreased spherical and chromatic lens aberrations and enhanced image contrast (16, 18).

The highest recorded resolutions at liquid helium temperatures have been attained in the course of this work. At 1.8° to 4.2°K, consistent resolutions of 8Å to 16Å have been achieved as compared with those of about 50Å at corresponding temperatures about a year ago (19, 20).

We have also built, but not yet tested, a superconducting objective lens of 100,000 ampere-turns designed for work in the superfluid helium cryostat of the high voltage microscope. Taken together with the results of recent studies involving specimen radiation damage at 3 MeV carried out in France (21) and in Japan (22), the advantages of superconducting lenses, low temperatures and high voltages which we first reported in 1970 (16) are becoming more apparent. Among these are the unmatched stability of lens excitation current and high voltage (5 kV to 10 MeV), correction of spherical and chromatic lens aberrations including trapped flux, phase zone apertures, and coherent electron beam point cathodes operating in cryogenic ultra-high vacuums to decisively reduce radiation damage, contamination and thermal noise while enhancing image quality with an optimized image intensifier operating at liquid helium temperatures.

III. Biological Applications

These improvements in instrumentation and preparation techniques have enabled us to examine the macromolecular organization of biological systems in far greater detail than ever before possible. In doing so, they have extended the commonly accepted applications of electron microscopy beyond its consideration as a tool for descriptive morphology in thin tissue sections to one of analytical importance.

A. Bacteriophage Ultrastructure

Modified negative staining techniques disclosed the protein substructure of viruses, and analytical correlation of the results with x-ray and biochemical data enabled us to deduce the actual arrangements of the proteins in various spherical virus particles in both the dried and native hydrated states (23). Using a special multiple-spraying device to achieve microdroplet cross-spraying, modified negative embedding preparations also proved useful in the study of these labile systems. Special thin fenestrated films without the usual supports were used to achieve the highest resolutions, supplemented by dialysis staining techniques.

Improved pointed filaments and a double-condenser system fitted with apertures of 50 μ to 20 μ permitted us to obtain microbeams of 0.1 μ to 2 μ diameter. This was particularly useful for electron diffraction studies of these organic samples. In addition, a special liquid nitrogen stage was employed to cool the thermally insulated specimen support from $-130°$ C to $-70°$ C. A special shielding device composed of several superimposed copper or platinum apertures protected the specimens from contamination.

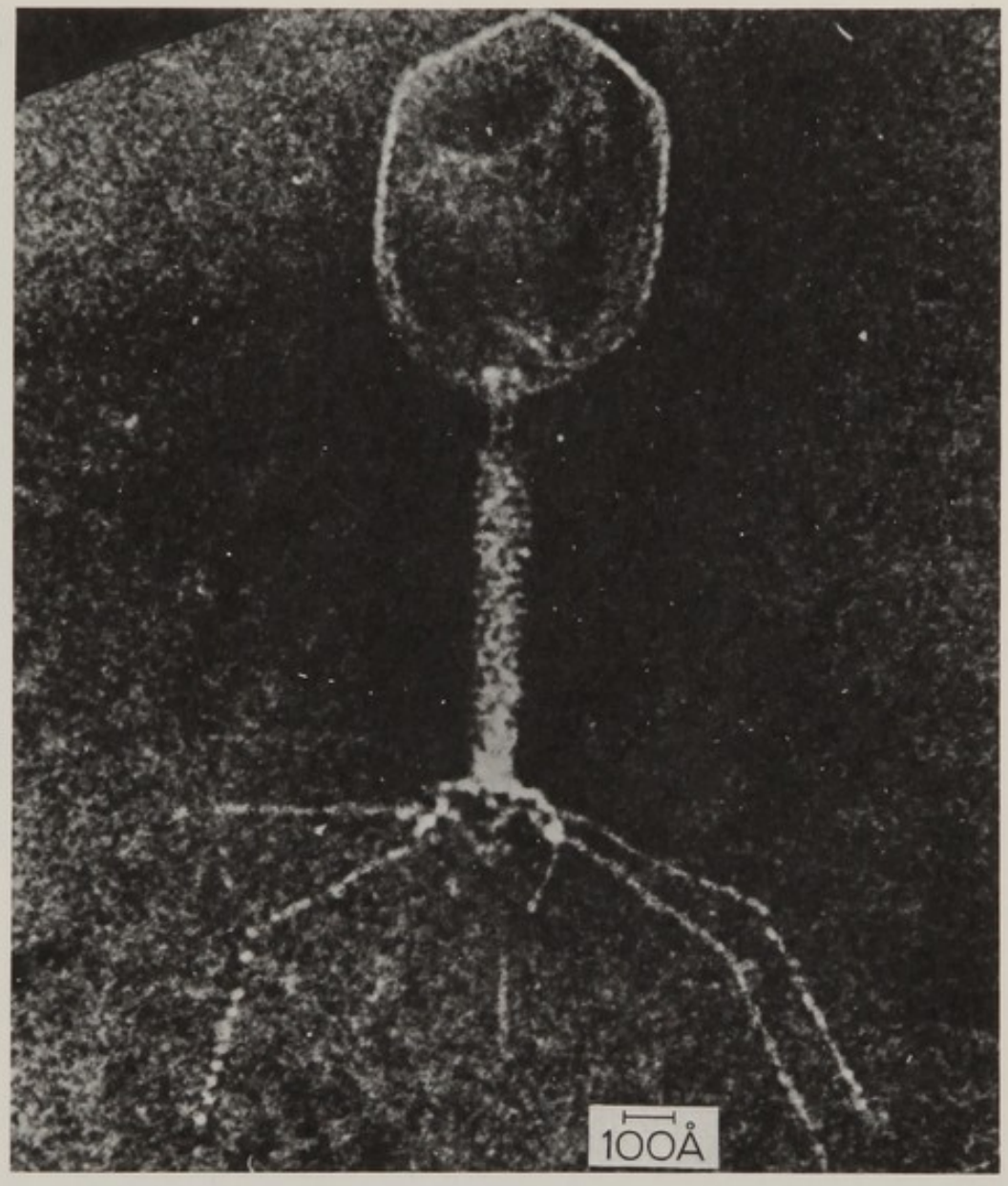

FIG. 1. High resolution electron micrograph of T2 bacteriophage showing fine structure of the head, periodic sheath striations, and of the tail-plate with attached fibers. Double-spray negative staining technique.

The T2 and T4 bacteriophages are among the most complex viruses yet studied. Using these procedures, we have observed that these bacterial viruses are characterized by proteins having different primary structures (23-25).

The T2 bacteriophage has a hexagonal head ca. 600Å to 800Å long. The tail is made up of rays symmetrically disposed around a central hole ca. 60Å to 80Å in diameter. Six kinked tail fibers (ca. 20Å wide and 1300Å long) are connected to the head by a coiled collar-type protein structure (figs. 1, 2). The fully extended complex has the characteristic

FIG. 2. Electron micrograph of the hexagonal base plate of T2 bacteriophage showing the characteristic 6-pointed star with symmetrically arranged tail fibers attached to the ray tips by dense knobs (kf). This labile tail-plate complex could only be observed in modified negative-staining preparations using microdroplet cross-spraying techniques.

shape of a 6-pointed star about 550Å to 600Å in diameter when isolated intact (fig. 2), and regular sheath striations (ca. 25 per sheath with spacings of 30Å to 35Å) can be clearly observed. The fine structure detected in the striations may be correlated with biochemical evidence which suggests that the sheath contains protein subunits of ca. 50,000 molecular weight.

The texture of the composite head membrane has also been studied, and primary protein structures have been observed with consistent resolutions of 10Å to 20Å at room temperatures.

B. DNA Molecules Studied in Solution

Electron microscope observations have been made of DNA molecules under conditions closely approximating those of the native hydrated state.

Specimens were enclosed in vacuum-tight microchambers with multiple cavities of 100Å to 1000Å (26) and examined with low intensity microbeam illumination.

In T2 bacteriophage DNA preparations, thin fibrils about 30 μ to 40 μ long could be seen. They appeared to be formed by unit filaments of 20Å diameter in an ordered lateral aggregation. Additionally, indications of axial discontinuities of approximately 30Å were clearly visible (27) (fig. 3).

Oriented DNA fibers were also mounted in vacuum-tight micro-

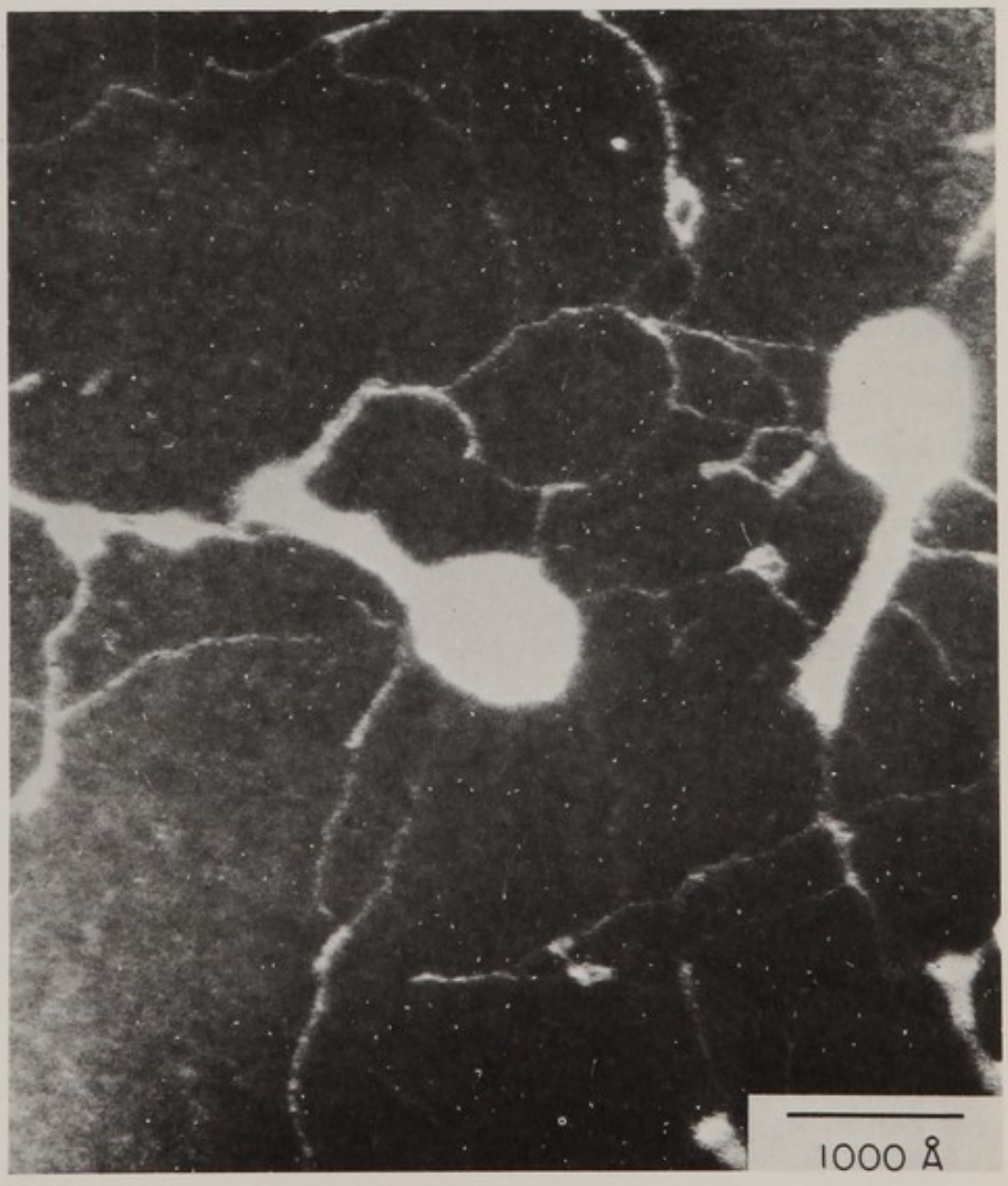

FIG. 3. Dark-field electron micrograph of T4 phage particles mounted on thin carbon substrate showing fine structure of released DNA strands. Recorded with modified HU-11 microscope using improved point cathode source, new thin-film annular condenser aperture and short-focal length (1.5 mm) objective lens at 75 kV.

chambers at controlled relative humidity in a form suitable for electron diffraction studies.

Irradiation with electron beams of higher intensity produced characteristic fragmentation of the filaments in hydrated DNA preparations. This differs markedly from the relatively stable appearance of the strands in dried specimens.

The improved pointed filament sources enabled us to restrict electron microbeam irradiation to accurately defined regions of 100Å to 1000Å along the fibrils. This controlled irradiation provides a promising tool for directly observing radiation damage in selected macromolecular regions of hydrated biological systems with the electron microscope.

C. High Voltage Studies of Whole Mount Preparations

Based on the pioneering work of Drs. G. DuPouy, F. Perrier and their colleagues in France, it has recently been pointed out that there are several advantages to high voltage electron microscopy from the biological scientist's viewpoint. Among these are (a) increased penetration power which permits examination of much thicker preparations and specimen whole mounts, (b) improved dark field ability due to the decreased chromatic aberration, and (c) reduction in spherical aberration which gives better resolution in bright field (28).

Recently, we have carried out systematic studies of whole mount unstained specimens of blue-green algae and phycovirus SM-1A particles prepared by J. MacKenzie using the modified Hitachi HU-200E microscope. Figures 4 and 5 show the same specimen area recorded at 50 kV and 200 kV. A remarkable difference is shown in the amount of detail which can be directly visualized as we move to higher voltages.

Point resolutions of 5Å to 10Å were observed in the 300Å to 500Å-thick specimens (19). In addition, we were able to directly reveal heretofore unavailable information of membrane and virus fine structure. Certain details of the viral infection process could be clearly detected, including different stages of virus integration with the host cell and alterations in viral structure during the course of infection. A comparison of figures 6 and 7, which show the same specimen area examined at 50 kV and 200 kV, demonstrates the advantages of high voltage penetration power in biological investigations.

By virtue of the higher resolution (ca. 6Å to 8Å) which we were able to achieve at 200 kV, this work supplements current research being carried out in France by DuPouy and his group using the far greater penetration power of the 3 MeV electron microscope. Corresponding investigations with a 3 MeV instrument are also underway in Japan.

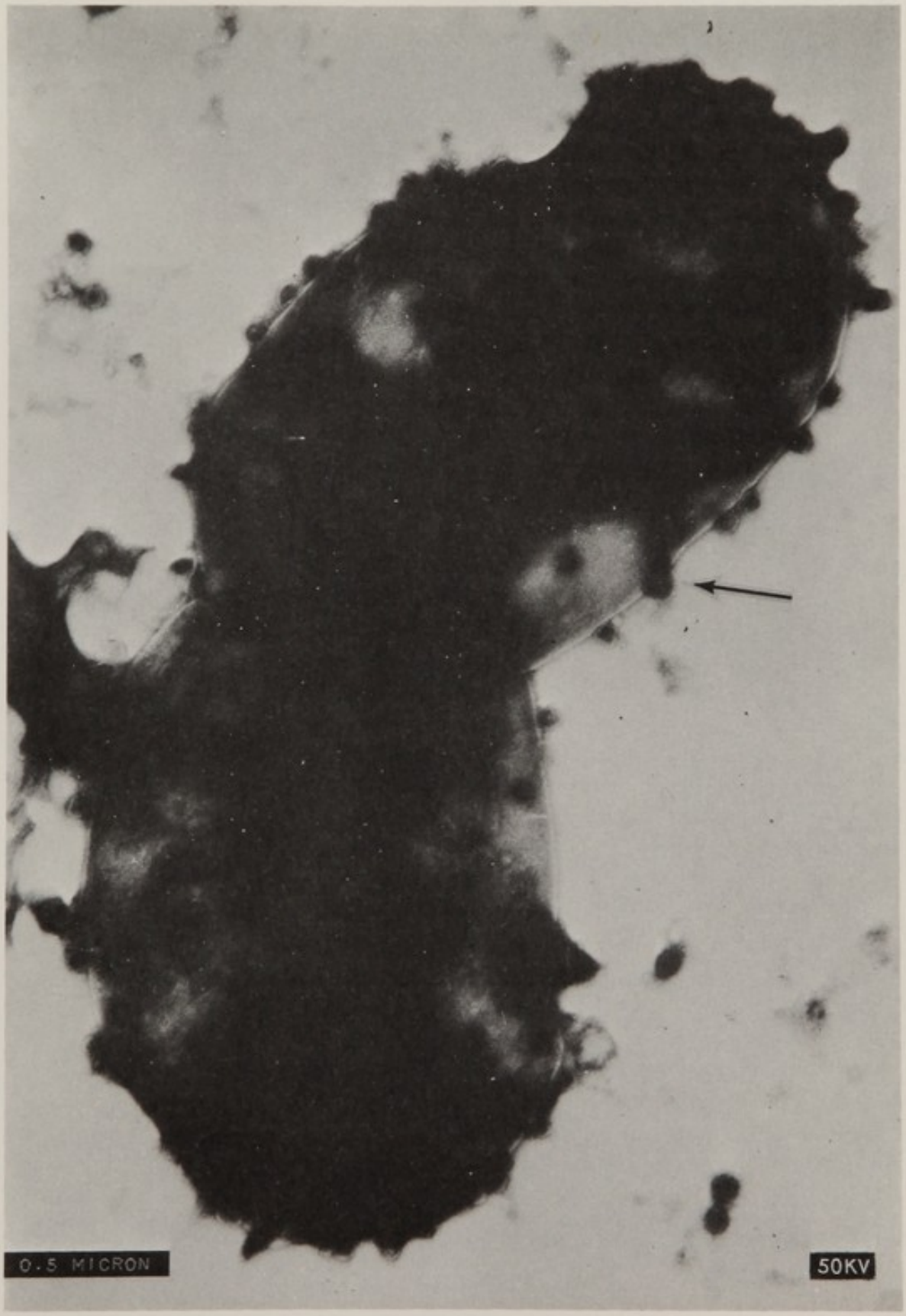

FIG. 4. Electron micrograph of phycovirus SM-1A infecting blue-green algae. This thick whole mount preparation recorded at 50 kV shows relatively little structural detail.

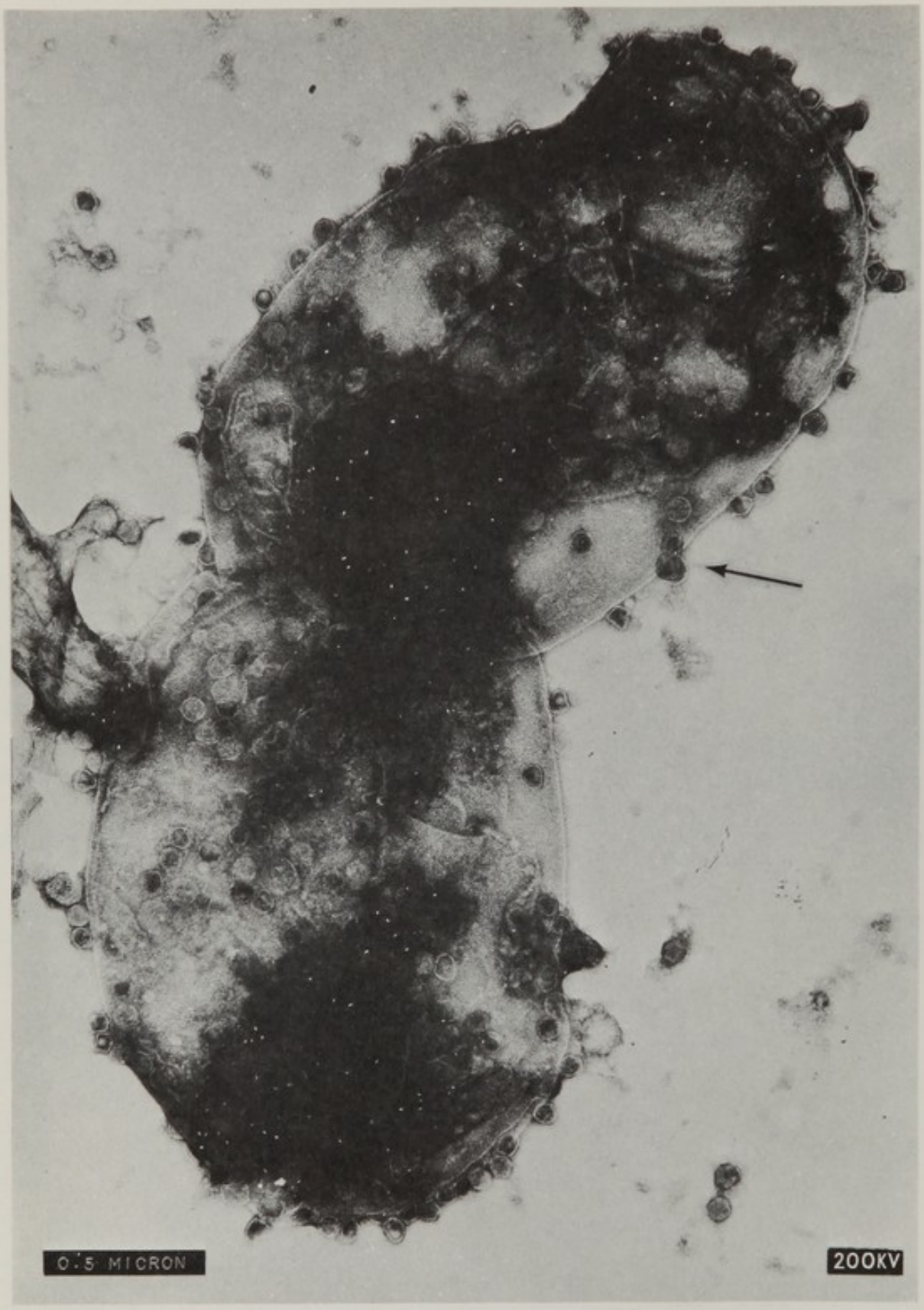

FIG. 5. High voltage (200 kV) electron micrograph of same specimen area in fig. 4 showing considerably enhanced penetration and resolution of new structural details of the virus-host cell interaction in this intact whole mount preparation.

FIG. 6. Electron micrograph of thick whole mount preparation of phycovirus SM-1A infecting blue-green alga recorded at 50 kV. Compare areas indicated by arrows with corresponding sites in fig. 7.

FIG. 7. Same specimen area depicted in fig. 6 now recorded at 200 kV showing characteristic electron transparency and enhanced resolution of virus fine structures (arrows) within the host cell.

D. RNA Polymerases and Related Enzymes

The enzyme RNA polymerase plays a key role in the transfer of genetic information through its participation in the differential RNA transcription upon DNA templates. Confirming the results of earlier workers, we have studied the physical properties of RNA polymerase molecules in *E. coli* (29).

A structure consisting of six subunits arranged in a hexagon with 120Å to 130Å cross-sections could be detected in negatively stained preparations of RNA polymerase molecules. A few smaller structures were also observed. These included square structures composed of four subunits closely resembling those found in the hexagons, and smaller (i.e., 70Å diameter) hexagonal structures (fig. 8).

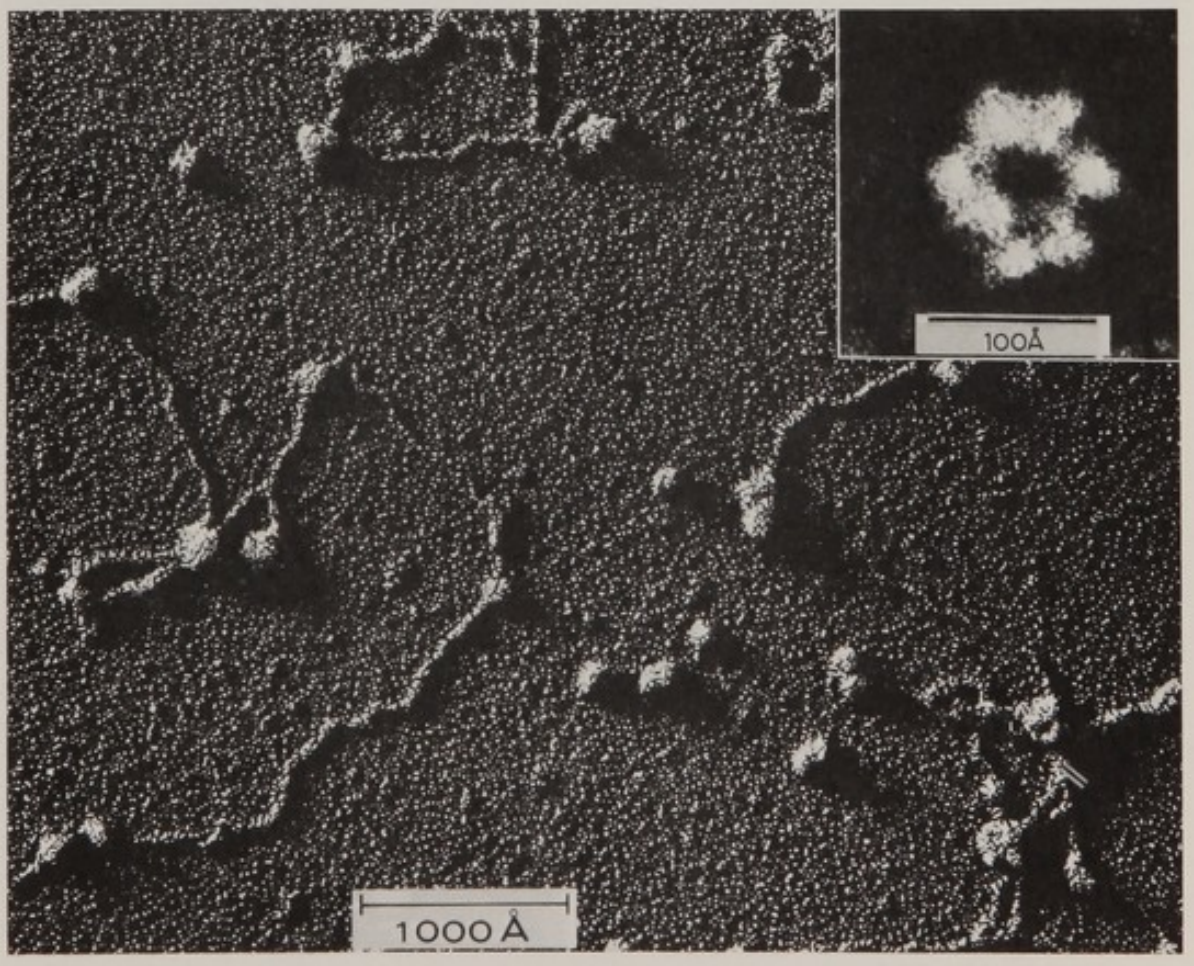

FIG. 8. RNA polymerase molecules attached to DNA strands in shadowed preparation. The insert shows negatively-stained DNA-dependent RNA polymerase molecules from *E. coli* prepared by the CHAMBERLIN and BERG procedure with six subunits in hexagonal arrangement.

In high protein concentration grid preparations, rectangular structures ca. 120Å wide and of varying lengths could be directly observed. These structures appear to consist of rows of three subunits each, and the rows seem to be arranged in pairs.

This suggests that if the large hexagonal structures are the RNA polymerase molecules, as has been hypothesized, the basic structure is either a hexagonal disc or, more likely, two of these stacked together. The smaller structures might well represent degradation or dissociation products.

In order to obtain more information on the manner in which RNA polymerase initiates transcription of RNA upon DNA templates, we have carried out correlated electron microscopic studies on the binding of RNA polymerase to different types of DNA. The attachment to circular forms of DNA (i.e., from ϕX 174) is of particular interest, and we have studied it together with R. GUMPORT and S. WEISS (19).

Since most naturally-occurring animal cancers to which viruses have been linked involve an RNA virus rather than a DNA one, the structural characterization of related enzymes takes on added importance.

In studies of tumor viruses, H. M. TEMIN and D. BALTIMORE independently observed that an enzyme, reverse transcriptase, uses RNA as a template for making DNA in the viruses. Subsequently, reverse transcriptase has been found in all RNA tumor viruses, and analysis for this enzyme has become part of the standard procedure in the study of suspected cancer-causing viruses (30). With this in mind, it would now be of great interest to examine the reverse transcriptase enzyme with the same electron microscopic techniques which have been used in the study of RNA polymerase.

E. Electron Microscopy of Hemagglutinin

Detailed studies of hemagglutinin from *L. polyphemus* conducted with G. EDELMAN and J. MARCHALONIS (31) resulted in the observation of macromolecular ring-shaped components with a diameter of ca. 100Å and an estimated height of 65Å (fig. 9). These structures can be differentiated from the hemocyanins and may represent another kind of protein in the hemolymph.

Each particle had a well-defined central dense core ca. 20Å to 40Å in diameter, and close examination of single molecules suggested that they had a hexagonal shape. Occasionally, we were able to visualize elongated structures 450Å by 100Å which seem to correspond to a side view of a stack of 100Å rings.

The dense central core could be further resolved into an axial cavity (ca. 15Å to 20Å diameter) lined by an electron-dense annular region

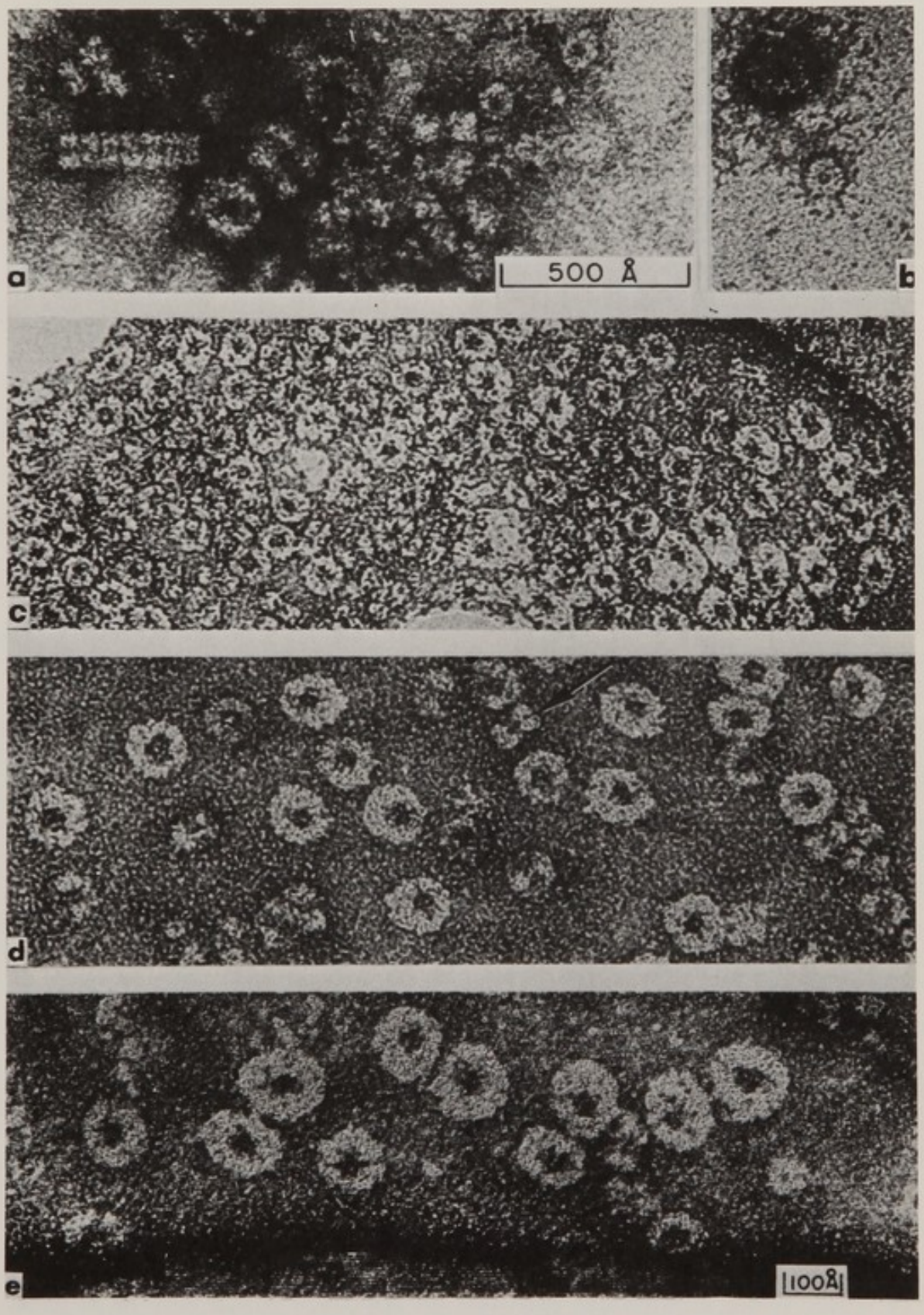

FIG. 9. High resolution electron micrographs of (a) fresh *Limulus* hemolymph stained with phosphotungstate, and uranyl acetate (b); (c) *Limulus* hemagglutinin prepared by J. MARCHALONIS and G. EDELMAN, stained with uranyl formate without prior fixation, and after fixation with glutaraldehyde (d) (e).

(ca. 40Å to 45Å in diameter). A doughnut-shaped ligther shell could be seen surrounding the core, apparently giving the particles their typical polygonal or hexagonal shape.

The detailed shape of the rings could not be completely defined, but the 6-fold symmetry with a hexagonal outline seen in some specimens was consistent with the proposed structure based on hydrodynamic studies, which consists of six units each composed of three subunits. Details of the subunit structure of the hemagglutinins could be visualized in some of the preparations examined, but their exact number and shape could not be fully determined.

By combining biochemical studies with the increased penetration power, high resolution and increased specimen preservation of the cryo-electron microscope and the uniformity of the hemagglutinin preparations, we can now begin to investigate hemagglutinin's mode of attachment to red blood cell membranes.

F. Oncogenic Viruses and Electron Microscopy

Viewing the hemagglutinin as a prototype antibody, this work is proving significant in the cancer research program. For example, Edelman has continued this research and found that certain proteins derived from green plants bind cancer cells into clumps, apparently by locking together the millions of cellular binding sites.

Recent investigations indicate that there is a direct connection between the membrane and the cancer-causing substance whose presence has been demonstrated through the use of mutant viruses (30). R. DULBECCO, W. ECKHARD and M. BURGER have shown that at high temperatures, when the product of the temperature-sensitive cancer gene is not working, the surfaces of the virus-infected cells are the same as those of normal cells. When the cancer protein or enzyme goes into action at lower temperatures, the cell surfaces acquire binding sites. While these sites exist in normal cells also, they are usually covered up and appear only during cell division. BURGER has proposed that surface alterations in the dividing cells, both normal and cancerous, serve as signals that set off synthesis of DNA in the nucleus. This, in turn, leads to cell division.

Electron microscopy has already proven ist unique capacity as a tool for directly visualizing the minute differences between virus particles and viral components. Our work with high voltage electron microscopy of whole tissue mounts has provided an approach which may be of great value in charting the hitherto unexplored areas, particularly in reference to the early stages of oncogenic viruses' interaction with host cells in controlled cell culture specimens.

Based on work reported here and on recent investigations into the relationship of cell membranes, viruses and cancer processes, we are beginning to carry out intensiye studies of the molecular organization of these organic systems in relation to the immunological processes, particularly the ultrastructural interactions involving cancer cell membranes and similar surface phenomena.

G. Dark Field Methods

Extending earlier work (26, 32), during the past year, the cryo-electron microscope and newly developed dark field methods have been used to carry out comparative studies of both dried and hydrated DNA specimens. Direct resolution of molecular and pauciatomic structures 4Å to 10Å have been achieved under ideal conditions at 290°K and at 1.8° to 4.2°K (17, 20).

These high resolution dark field studies have also demonstrated that unstained specimens when shadow cast with thin (ca. 50Å) grainless carbon films reveal new details which cannot be seen in corresponding shadowed bright field images. Figures 10 and 11, taken by M. OHTSUKI, provide a comparison of the same DNA specimen area in both bright and dark field. This is an important development in that it promises to yield the highest attainable instrumental resolutions (ca. 3Å to 7Å) while at the same time partially protecting the biological specimens against radiation damage (33).

Even allowing for unavoidable radiation damage such as chemical deterioration and its accompanying morphological changes, native molecular structure, or its meaningful equivalent picture, can still be recorded in the form of grainless replications obtained by shadow casting with carbon films prior to electron microscopic observation.

H. Hydrated Membrane Structure

Correlated ultrastructural and biochemical studies over the past few years have revealed certain general characteristics of membrane organization. Coherent paucimolecular layers of indefinite lateral extension appear to consist of a periodic, hydrated lipoprotein substrate which is integrated with specific macromolecular repeating subunits organized in asymmetric paracrystalline arrays within the plane of the layers.

Our work with DAVID GREEN and his associates (34) has demonstrated that membranes are made up by the stereospecific association of macromolecular repeating units of lipoprotein whose conformation are dependent on their association with the membrane substrate. Many lipo-

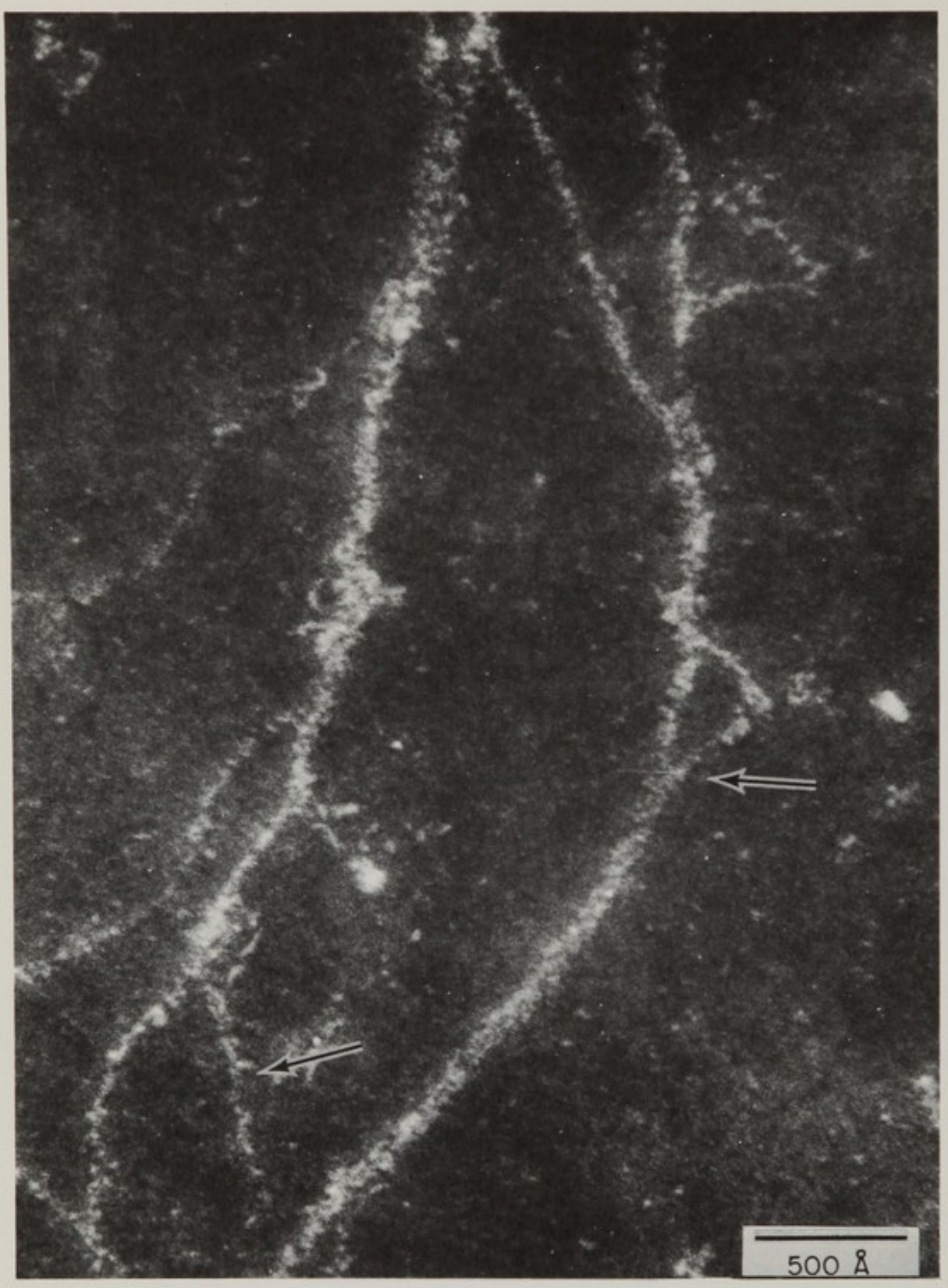

FIG. 10. High resolution dark-field electron micrograph of DNA from T4 phage mounted on special ultrathin carbon film (ca. 50Å) and lightly stained with uranyl formate. Notice the enhanced contrast and fine structural details of the DNA (arrows) revealed by these improved techniques. Recorded by M. OHTSUKI with modified HU-11 microscope using improved point cathode source, thin-film annular condenser aperture and short-focal length (1.5 mm) objective lens at 75 kV.

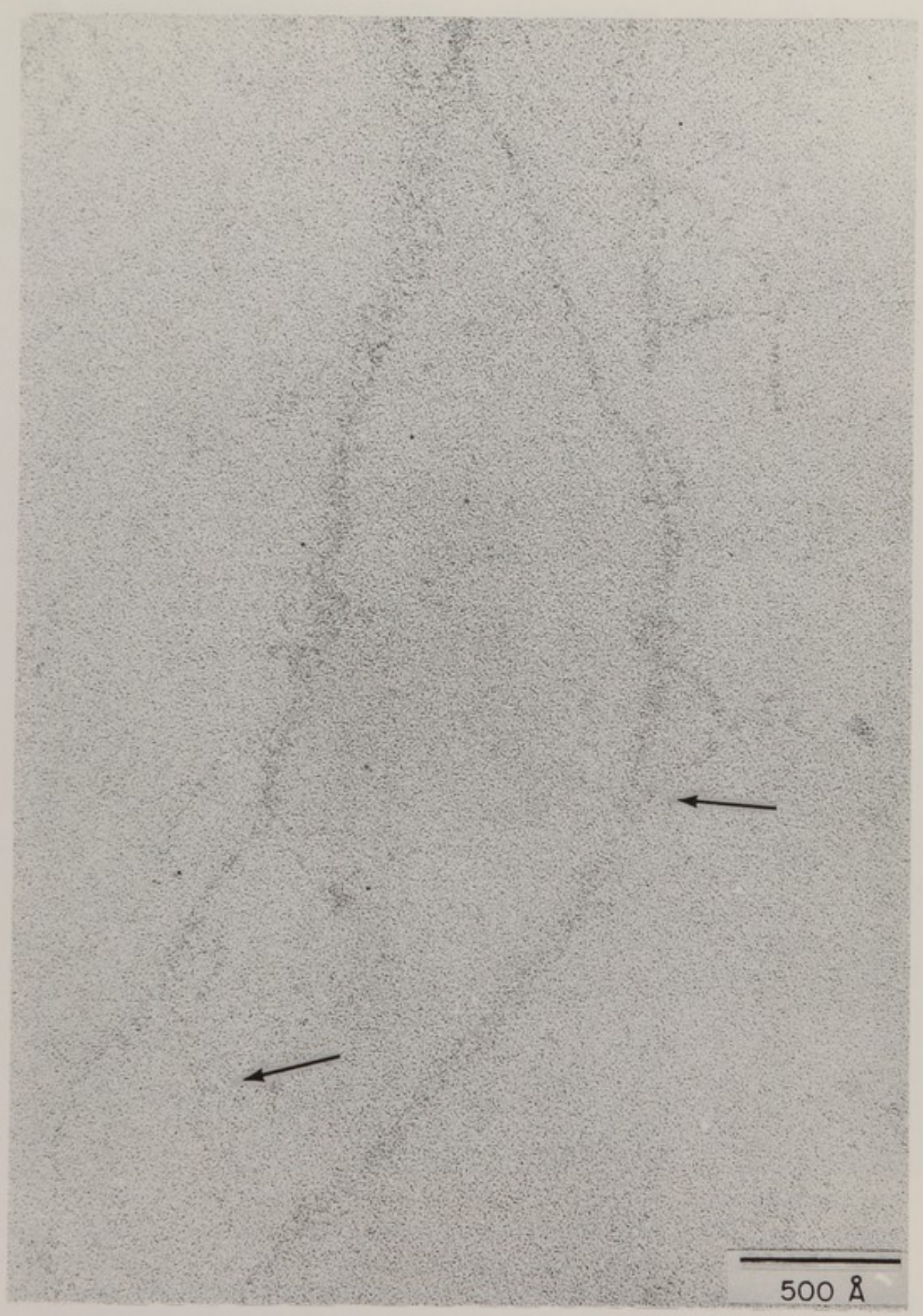

FIG. 11. Bright-field image of same specimen area shown in fig. 10 recorded under identical conditions. Compare the reduced contrast and the distinct background structure which obscures details of the DNA fine structure (arrows).

protein membranes, both native and artificial, respond *in vivo* and *in vitro* to the binding of specific ligands by some modification of their properties. This process reflects the rearrangement of membrane organization and, presumably, of the repeating unit conformation (19, 32).

Systematically applying high voltage cryo-electron microscopy, diamond knife cyro-ultramicrotomy and related electron optical developments, we have been able to approach the native hydrated state in investigations of various biological specimens, directly observing increasing amounts of meaningful information and structural detail (17-20).

Specimens of DNA, nerve myelin, cock retinal rod outer segments and bacteriophages have been sandwiched between impermeable ultrathin films in specially-adapted vacuum-tight microchambers (26) to trap their hydrated components at liquid helium temperatures. Data indicate that new details of hydrated membrane structure and dimensions can be visualized with resolutions of ca. 8Å to 16Å (fig. 12). While these results are only preliminary in nature and are still being evaluated, they do indicate the potential of the cryo-electron microscope in biological and biomedical studies of hydrated systems.

IV. Outlook for the Future

Rather than viewing the membrane as a static structure, EDELMAN has characterized it as a dynamic sheath in which specific molecules are sticking out and moving about. We are now just beginning to study the functional relationships between these structures. Indeed, the electron microscope and the high voltage cryo-electron microscope have opened the whole new field of hydrated cell and virus ultrastructure.

Without the electron microscope, would we have been able even to imagine virus complexity to the degree shown in the T2 phage? This is significant since, remembering the size of the virus particle, every atom in its structure counts and has its own function. Now we are on the verge of correlating structure and function in this pauciatomic domain.

Correlated cryo-electron microscope and biochemical studies are beginning to give researchers some idea of how the virus interacts with the infected cell in a way that biochemistry and x-ray studies alone cannot approach. Through direct visualization under native state conditions, we are learning more about DNA, RNA, RNA polymerase, reverse transcriptase and other enzymes without which viruses cannot function.

These steps toward building a meaningful understanding of the processes and structures involved in living systems are important ones, but the full potential of the electron microscope is still to be realized. Looking to the future, it appears most profitable to concentrate on certain key problems of membrane and viral ultrastructure:

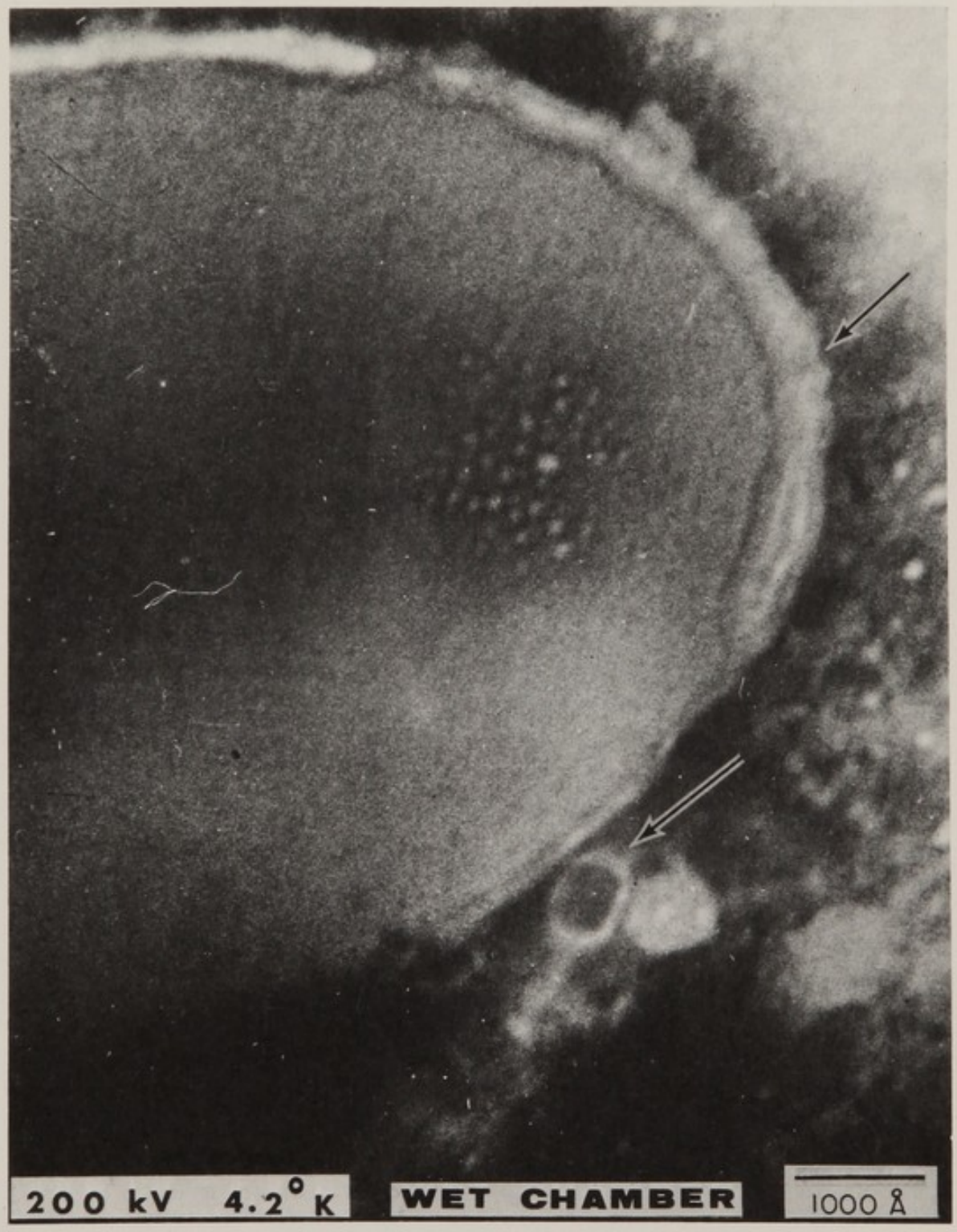

FIG. 12. High resolution electron micrograph of supercooled bacteria and bacteriophage examined with 200 kV cryo-electron microscope with specimen cooling at 4.2°K in a specially-designed vacuum-tight microchamber. New details of hydrated bacteriophage and membrane structures (arrows) can be observed in these relatively thick specimens with resolutions of ca. 10Å to 20Å.

a. Correlated biochemical, electron microscopic and biophysical characterization of the major protein, lipoprotein, glycoprotein and nucleoprotein structures within the virus and the host cell in a variety of specimens, including oncogenic viruses.

b. Systematic studies of the organization of virus subunits in the native hydrated state under conditions of reduced radiation damage, contamination and thermal noise using improved instrumentation and preparation techniques for high resolution, low-temperature electron microscopy correlated with polarized light and x-ray diffraction studies.

c. Correlation of ultrastructural studies with a comprehensive macromolecular and molecular anatomy program aimed at achieving reliable dissection and quantitative separation of the entire spectrum of discrete viral species using ANDERSON'S zonal centrifuge techniques (35) and related methods.

d. Correlative studies of specific, dynamic changes of selected key sites of cancer tissues *in vivo* and *in vitro* using light and electron microscopy.

It would be of special interest to carry out a correlated ultrastructural and biochemical analysis of the molecular organization of selected key sites of cancer tissues by performing a series of submicroscopic or macromolecular biopsies. Such biopsies could be carried out *in vivo* with specially developed ultra-microcapillaries, and they could then be combined with subsequent tissue culture explants and related *in vitro* techniques. HYDÉN (36) has already carried out significant pioneering work in microbiopsies of living tissues, and further development of these techniques holds a great potential for electron microscopy of oncogenic viruses.

New techniques for high resolution electron microscopy and diffraction at high voltages and liquid helium temperatures offer a singular opportunity to move us closer to these goals (1). By combining a high degree of both direct resolution and specimen preservation under native state conditions, these approaches may well hold the key to new insights into mechanisms of replication fundamental to all forms of life, including the borrowed life of viruses.

V. Acknowledgments

The research reported here represents a continous effort spanning over a decade in which I have been privileged and grateful to work with Prof. SAMUEL C. COLLINS of M.I.T. and the Naval Research Laboratory, Dr. F. O. SCHMITT of M.I.T., Dr. W. SWEET of the Massachusetts General Hospital, Drs. T. HALPERN, H. STANLEY BENNETT, W. BLOOM, D. YADA, R. ZIRKLE, L. MEYER, G. EDELMAN, J. MARCHALONIS, R. GUMPORT, S. WEISS, A. COLVILL and E. VAN BRUGGEN, all associated with the University of Chicago during the course of this work, Mr. R. SZARA and his associates of the Low Temperature Department, Dr. D. COHEN of the National Magnet Laboratory, Dr. C. LAVERICK of Argonne National Laboratory, Messrs. M. STREETER and R. OSBURN of Cryogenic Technology, Inc. These men provided helpful suggestions and assistance in the development of improved instrumentation, particularly superconducting lenses, the Collins closed-cycle superfluid helium refrigerator and the integrated cryo-electron microscope.

The major portion of the development and research has been carried out in a systematic team effort by the members of our interdisciplinary research staff, particularly M. OHTSUKI, C. HOUGH, H. KREBS, R. MOSES, R. VICARIO, G. ARCURI, J. RICHARDSON and G. BOWIE, together with the skilled men of the Physical Sciences Developmental Workshop. I wish to thank S. ROWE for the compilation and technical editing of this manuscript, S. ERIKSON for administrative assistance, and the many outstanding men and women who have been associated with our laboratory during the course of this research.

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THE MOLECULAR BIOLOGY OF A DEFECTIVE VIRUS

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CONTENTS

The molecular biology of a defective virus

I. Introduction

Many viral preparations contain defective particles which interfere with the replication of their related non-defective standard viruses. Homologous interference caused by these defective interfering (DI) particles have been reported for every major group of animal viruses except herpesviruses and poxviruses (see HUANG, 1973). This interference by defective particles has been proposed as an important mechanism operative during viral pathogenesis (HUANG and BALTIMORE, 1970).

By definition DI particles 1) contain a part of the viral genome, 2) contain normal viral structural proteins, 3) reproduce only with the help of standard virus and 4) interfere with the growth of standard virus (HUANG and BALTIMORE, 1970). Interference by DI particles 1) occurs intracellulary, 2) is specific for the homologous viral system, 3) is destroyed by prior ultraviolet-irradiation of DI particles and 4) alters the synthesis of virus-specific, intracellular nucleic acids.

This paper summarizes data from the vesicular stomatitis virus (VSV) system to illustrate the properties of DI particles. The first separation and positive identification of DI particles was with VSV (HUANG *et al.*, 1966; HACKETT *et al.*, 1967) and studies on VSV have provided information on the genesis of DI particles, their physical and biological properties and the molecular basis of interference.

VSV is a large enveloped RNA virus which is shaped like a bullet (HOWATSON and WHITMORE, 1962; PREVEC and WHITMORE, 1963). It contains single-stranded RNA which has a sedimentation constant of 40S (HUANG and WAGNER, 1966b; STAMPFER *et al.*, 1969) and has a virion-associated polymerase which transcribes the virion RNA into complementary RNA (BALTIMORE *et al.*, 1970). The multiplication of the virus occurs in the cytoplasm and is independent of cellular DNA-dependent RNA synthesis (PREVEC and WHITMORE, 1963).

II. VSV DI Particles

There have been several types of DI particles isolated from the VSV system. The wild-type Indiana serotype of VSV produces DI particles one-third and two-thirds of the length of standard virus. These particles have been called T particles and LT particles, respectively (HUANG *et al.*, 1966; PETRIC and PREVEC, 1970). A heat resistant strain of the same serotype produces only LT particles (PETRIC and PREVEC, 1970). Temperature-sensitive mutants of the Indiana serotype each generate a specific DI particle even after repeated clonal isolations (REICHMANN *et al.*, 1971). In contrast to these DI particles of rather unique lengths, the New Jersey serotype of VSV usually generates a heterogenous group of DI particles which are difficult to separate from standard virions (HACKETT *et al.*, 1967). The most extensively studied type of DI particle has been the T particle of VSV. Most of the following data have been obtained with T particles.

III. Properties of the Defective T Particles of VSV

Separation of T particles from the larger plaque forming B particles was accomplished by rate zonal centrifugation in sucrose gradients (HUANG *et al.*, 1966; HACKETT *et al.*, 1967). T particles contain one-third the genome of B particles and are one-third the length of B particles, but in every ultrastructural detail the two types of particles are identical (fig. 1). Polypeptide analysis of T and B particles shows that the two particles contain identical structural proteins (WAGNER *et al.*, 1969; KANG and PREVEC, 1969). T particles are also capable of eliciting neutralizing antibodies when injected into rabbits and fix complement with antiserum specifically directed against B particles (HUANG *et al.*, 1966).

Proof that the RNA of T particles is an unique third of the genome of B particles comes from hybridizing the RNA of T particles to specific classes of complementary VSV-specific RNA, either obtained from infected cells or produced by the virion-associated polymerase (SCHINCARIOL and HOWATSON, 1972; ROY and BISHOP, 1972). The RNA of T particles has deleted the sites for initiation of transcription by the virion-associated enzyme and therefore, T particles *in vivo* neither synthesize messenger RNA nor viral proteins (ROY and BISHOP, 1972; EMERSON and WAGNER, 1972; HUANG and MANDERS, 1972). Also, T particles do not complement temperature-sensitive VSV mutants (REICHMANN *et al.*, 1972). The genome of T particles is minimally functional in that it is capable of only replicating and interacting with structural proteins to form a stable virion.

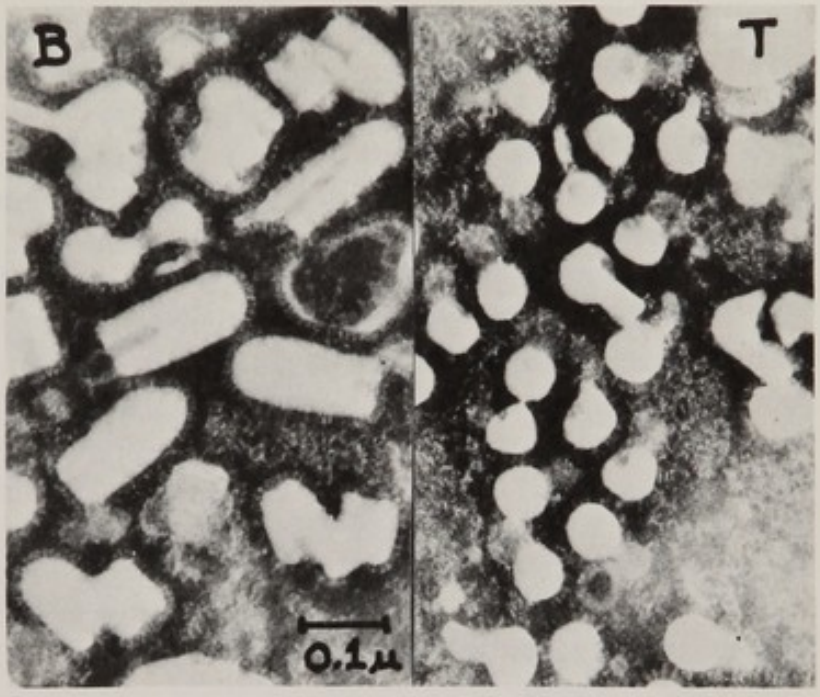

FIG. 1. Morphology of B and T particles, stained with phosphotungstic acid. These photographs were kindly taken by Dr. JOHN W. GREENAWALT (HUANG *et al.*, 1966).

IV. Genesis of T Particles

How such a deletion occurs to from the RNA of T particles is not well understood. Careful cloning of VSV through several successive plaque isolations has shown that viral preparations can be obtained free of any DI particles (STAMPFER *et al.*, 1971). Upon subsequent passage the population of virions becomes more heterogenous with the appearance of DI particles. VSV is classed as a high frequency virus system for the formation of DI particles, because they are usually detected around the third to fourth passage after cloning. Also, DI particles of VSV are synthesized readily in almost every type of cell (PERRAULT and HOLLAND, 1972a). Because detection of DI particles is based on the relatively insensitive method of radioisotope incorporation into DI particles, a mutation rate for the initial appearance of one DI particle cannot be measured.

A most sensitive way to detect the presence of T particles in VSV preparations is by observing the synthesis of intracellular virus-specific RNA. The shift of synthesis from RNA species, called group I, to RNA species, called group II, is diagnostic of the presence of T particles even when T particles are not observed by any other means (STAMPFER *et al.*, 1969; fig. 2).

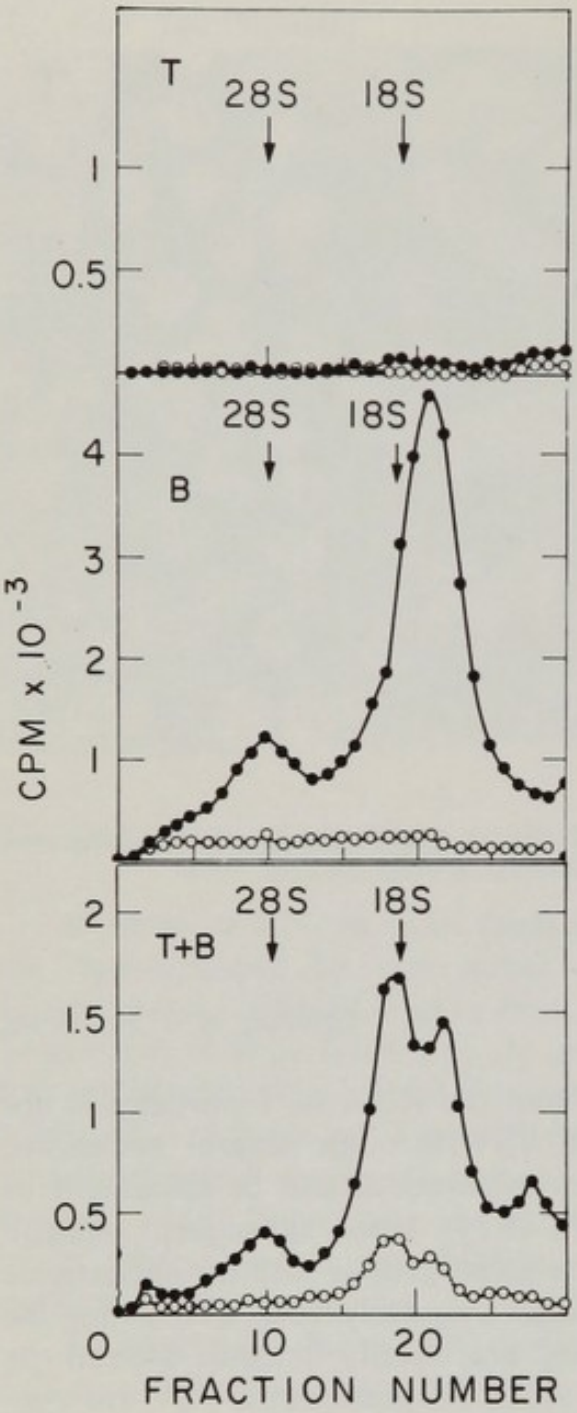

FIG. 2. Sucrose gradient patterns of VSV-specific RNA synthesized in Chinese hamster ovary cells infected by T particles alone, B particles alone, and T and B particles. Concentration of T particles was enough to inhibit the growth of B particles by 99.99% and the concentration of B particles used was at a multiplicity of 1. These experiments were performed by Dr. MARTHA STAMPFER as previously described (STAMPFER *et al.*, 1969). Filled circles= total acid-precipitable ^{14}C-uridine; open circles= ribonuclease-resistant, acid-precipitable ^{14}C-uridine.

V. Interference

Interference with the growth of B particles by T particles is intracellular because addition of T particles after B particles have attached

and penetrated into cells still inhibits the multiplication of B particles to the same degree as addition of B and T particles to cells at the same time. The interference is not mediated through the production of interferon because interference by T particles occurs in the presence of actinomycin D (STAMPFER *et al.*, 1969). Also, T particles inhibit only B particles of the Indiana serotype. Interference against the New Jersey serotype is one-hundred times less effective. Against encephalomyocarditis virus, T particles have no effect at all (HUANG and WAGNER, 1966a).

The specific ability of T particles to inhibit the production of B particles is related to the ability of T particle RNA to be replicated. Ultraviolet-irradiation of T particles destroys their interfering properties (HUANG and WAGNER, 1966a). Also, for interference to occur, it must occur relatively soon after the initiation of infection by B particles because super-infection of cells with T particles 2-3 hours after B particles results in much less inhibition of B particles than when T particles are added to cells at the same time as B particles.

The first synthetic step that B particles perform in cells is complete transcription of the genome into complementary messenger RNA (HUANG and MANDERS, 1972). This step is not inhibited by the presence of T particles (HUANG and MANDERS, 1972; PERRAULT and HOLLAND, 1972b). Also, viral protein synthesis is not inhibited by the presence of T particles because T particles have no effect on the synthesis of B particles if added late in infection.

When cells are infected with VSV and the production of B particles is almost completely inhibited by T particles, there is transcription resulting in VSV-specific messenger RNA and presumably viral proteins. The only RNA species which is replicated is the RNA of T particles (HUANG and MANDERS, 1972). Such cells are not killed as rapidly as cells infected with B particles alone, and the cell surface is altered to resist super-infection by other B particles (PALMA and HUANG, unpublished observations). These observations lead to two interpretations: 1) T particles inhibit the synthesis of B particles during virion RNA replication and not transcription and 2) a secondary type of interference occurs at the cell surface with cells previously infected with B and T particles.

Under conditions of interference when a large amount of T particles are being produced, there is, also, intracellular retention of nucleocapsids, sometimes consisting of about 80% of the total intracellular VSV-specific RNA (PALMA and HUANG, unpublished observations; KANG and PREVEC, 1971). In contrast, when interference is not occurring, nucleocapsids represent 10% or less of the total intracellular viral RNA. It is suspected that the accumulation of large amounts of intracellular nucleocapsids is not due to a maturational defect in morphogenesis caused directly by DI particles but is due to the indirect effects of DI particles on RNA replication. Neverthless, such accumulation represent inclusion bodies and are

diagnostic of interference caused by DI particles. Further studies on these aspects of interference will lead us to an understanding of the molecular basis of interference by DI particles.

VI. Cyclic Production of Viral Particles

During continued passage of animal viruses in cell cultures, or in persistently infected cultures, many investigators have noticed a cyclic variation in the viral titers. In particular, WAGNER *et al.* (1963) established a persistently infected culture of L cells with a small plaque variant of VSV, which occasionally, over a six-month period, caused complete destruction of the cells accompanied by a rise in viral titer. Recent studies with B and T particles show that this variation can be due to T particles (PALMA and HUANG, unpublished observations). When radioactive B and T particles are measured during continuous passage, there is an overlapping cyclic production of B virions and T particles (fig. 3).

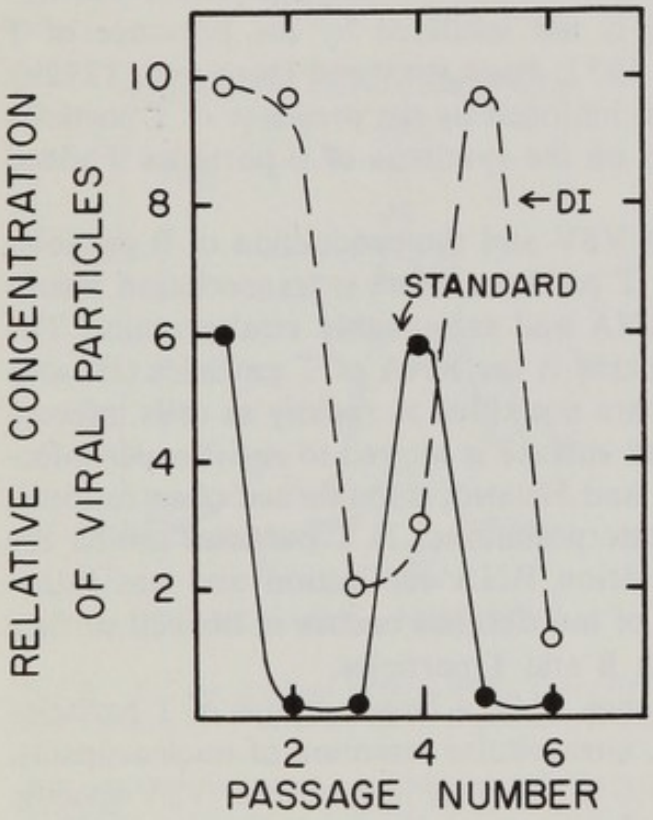

FIG. 3. Relative production of B (standard) and T (DI) particles by continuous infection of Chinese hamster ovary cells. Cells infected with B and T particles were incubated at 37 °C and mixed with uninfected cells every 24 hours in a ratio of 1:10. An aliquot of each passage was withdrawn and treated with ^{3}H-uridine (New England Nuclear, >21 C/mM) and actinomycin D (kindly supplied by Merck Sharp and Dohme). Extracellular labeled B and T particles were assayed by measuring the RNA species or the nucleocapsid structures in sucrose gradients as previously described (STAMPFER *et al.*, 1969; HUANG *et al.*, 1970). These experiments were performed by Dr. EDUARDO L. PALMA.

Interdependence between defective particles which are helper-dependent and standard virions which are susceptible to inhibition by defective particles results in non-coincident oscillating patterns representing the production of DI particles and standard virions. The oscillations representing DI particles lag slightly behind those representing standard virions. The frequency and amplitude of the oscillations can vary. One of the factors affecting the relative amounts of T and B particles is the species of host cells (HUANG and BALTIMORE, 1970). It will be of interest to determine what other factors will alter the pattern of production of the two types of viral particles.

Undoubtedly, further studies on the molecular biology of interference by defective particles of VSV will not only be useful in understanding macromolecular synthesis and control, but also the relation between DI particles and the pathogenesis of infectious viral diseases.

VII. Acknowledgments

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MOLECULAR BIOLOGY OF TOGAVIRUSES[a]

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CONTENTS

Molecular biology of togaviruses*

I. Introduction

The genus Togavirus has now been proposed to cover the great majority of arthropod-borne viruses (arboviruses) belonging to the serological groups A and B. They are usually referred to as the "typical" or "true" arboviruses. The *Vertebrate Virus Subcommittee* of the International Committee on Nomenclature of Viruses suggested to use the term arbovirus only in a purely biological sense and as an epidemiological designation, and to create the genus togavirus in order to designate a more homogenous group of arboviruses (1). Members of the togavirus group have similar physical chemical and morphological properties, such as: the structure of the virion and its symmetry, chemical composition, and the site of replication and maturation. The general characteristics of the genus are: the presence of nucleic acid of the RNA type, cubic symmetry and the presence of a lipo-protein envelope, regardless of whether they are or are not arthropod-borne. A detailed description of their properties and their place in the universal system of virus classification can be found in a recent review by J. L. MELNICK (2).

The togavirus group is of special interest to the modern virologist and molecular biologist for a number of reasons, as follows: *(a)* It is the first larger group (some 60 virus types) of viruses which have been delineated from the heterogenous conglomerate of arboviruses. *(b)* Recent studies on the structure and biochemistry of the togavirion indicate that, although the biogenesis and morphogenesis of these viruses are complex, their biochemical composition is relatively "simple"; all members of the togavirus groups studied to date are composed of two, and at most, three polypeptides. One of these structural proteins is in the viral envelope, the

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other, in the nucleocapsid. *(c)* Since all, or most, of the antigenic properties are contained in the viral envelope, it is possible to study the relationship between virus structure and composition, and "immunological" function.

II. Viral Replication

The scheme of replication of the togaviruses has not yet been elucidated in spite of the fact that during the last five to six years extensive studies of their mode of replication have been carried out. Most of these investigations are concerned with members of Group A togaviruses and only a few studies were done with members of Group B. The great majority of the reports deal with a limited number of A togavirus species: Semliki Forest, Sindbis, and Western Equine and Eastern Equine Encephalitis viruses.

All earlier reports (3-7) indicate that during infection of susceptible cells in culture with the various members of Group A togaviruses, a number of different species of viral RNA can be demonstrated, as follows:

1. A 40S to 45S RNA. This is a single-stranded, infectious and, probably non-segmented, RNA molecule. It is found in the infected cell and accounts for 99% of the infectivity. An identical RNA molecule can be extracted from the mature purified virion.
2. A double-stranded (or multi-stranded) 16S to 20S form. It is non-infectious and ribonuclease-resistant. This RNA species is possibly a mixture of what is referred to in picornaviruses as the replicative form (RF) and replicative intermediate (RI). The techniques of sucrose gradient centrifugation—which were, until recently, mainly used for the separation of the various viral RNA species—did not enable the distinction of the RF and RI. Only recently claims were made that the RF and RI of Semliki Forest and Sindbis virus can be adequately separated by the use of high resolution polyacrylamide gel electrophoresis (8).
3. A single-stranded, very poorly infectious ribonuclease sensitive 26S to 27S RNA. This species of RNA, also referred to as "interjacent" RNA is unique for the togavirus group. It is rather difficult to identify and recognize because it has no known biological properties by which it can be measured. Very little infectivity (ca. 1%) is associated with it. According to DOBOS and FAULKNER (9), 26S RNA of Sindbis virus behaves in polyacrylamide gel electrophoresis as a heterogenous population of RNA molecules of different sizes and shapes. SREEVALSAN *et al.* (10) raise the possibility that 26S RNA of Western Equine

Encephalomyelitis virus may be a transitional form of RNA, leading to the fully infectious 42S molecule.

The precise nature, function and interrelationship of the different RNA species isolated from cells infected with the various A group togaviruses is not well established. Pulse labelling experiments have indicated that the earliest form to appear is the 20S. This double—or multi-stranded form is initially formed in the infected cell. This is followed very closely by the appearance of the single-stranded 26S RNA. The last to appear is the 42S RNA species.

The properties of and interrelationship between the 40S-42S and 26S RNA species have been studied extensively (9-11). The 42S RNA of Sindbis virus, obtained from purified virions or extracted from infected cells, can be converted to 26S RNA by heating to 85 °C for a short period of time. The "naturally occurring" 26S RNA (isolated from cells infected with Sindbis virus) and the 26S RNA "derived" from 42S RNA exhibited complete similarity in electrophoretic pattern. The 40S Western Equine Encephalitis RNA can also be converted to 26S RNA by heating and quick cooling, as well as by treatment with 8M urea. Such denatured RNA loses over 90% of its infectivity. Basically these two forms of RNA are similar in size and structure and they have identical base compositions and densities. It is thus suggested that these two RNA species differ only in their secondary structure. It is not yet established, however, whether 26S RNA is a precursor of 42S.

In order to study the details of viral RNA synthesis, a number of studies were carried out with *in vitro* viral polymerase systems. MARTIN and SONNABEND (12) demonstrated that a Semliki Forest virus specific polymerase can be isolated from infected cells during the time of rapid viral RNA synthesis. This enzyme catalyzed *in vitro* the synthesis of a single viral RNA species, i.e. a ribonuclease-resistant RNA which had the physical chemical properties of the double-stranded 20S RNA found in infected cells. Repeated attempts failed to demonstrate the synthesis of any species of single-stranded RNA. SREEVALSAN and FAY HOH YIN (13) isolated an RNA polymerase from Sindbis virus-infected cells which synthesized, in addition to the ribonuclease-resistant 20S RNA, a single-stranded 40S RNA species.

III. Site of Viral Replication and the Morphogenesis of the Virions

One of the earliest thorough electron microscopic studies of the structure and development of a togavirus was done by MORGAN *et al.* (14). These authors described the various stages in the development and release of Western Equine Encephalomyelitis virus in chicken embryo fibroblasts

in culture. They demonstrated that "precursor particles" (nucleoids), 22 nm in diameter, differentiate at template sites in close proximity to cell membranes bordering cytoplasmic vacuoles. Mature virus is formed by a budding process across cellular membranes. The nucleoids acquire in this process a coat, and they are then extruded on the cell surface as mature virus particles. ACHESON and TAMM (15) have studied with the electron microscope the replication of Semliki Forest virus in chicken embryo cells. The electron microscopy findings were correlated with the kinetics of production of infectious virus. They found that free virus nucleoids are found scattered in the cytoplasm during the period of rapid virus production. The nucleoids migrate to the plasma membrane where mature virus particles are formed by a budding process. Evidence was obtained that, during viral assembly, the envelope which surrounds the viral core consists of a portion of the plasma membrane which has become covered with projections. Further studies of the site of replication of group A togaviruses have confirmed and extended these former findings (7, 16-20). Formation of unique cyptoplasmic structures, designated type I cytophatic vacuoles (CPV-I) was demonstrated early in the infectious cycle of Semliki Forest virus by FRIEDMAN *et al.* (17). Using autoradiography, these authors have shown that the CPV-I are the loci of active incorporation of ^{3}H-uridine. BEN-ISHAI *et al.* (7) have also provided biochemical evidence that the site of viral RNA and coat protein synthesis of Sindbis virus is localized in the cytoplasmic reticulum. Electron microscopic studies of the development of various members of togavirus Group A in mouse brain (18-20) have provided further evidence of a general similarity in the sequence of arbovirus maturation. The assembly of mature virus particles takes place by a process of budding of the virus precursors from the plasma membrane into the extra-cellular space, and also into the lumina of vacuoles and cisternae. These findings support previous evidence, obtained from togaviruses Group A grown in cell cultures, of a uniform mechanism of virus assembly: preformed, intracytoplasmic nucleocapsids acquire envelopes from the cell membrane, thus forming mature particles which are released into intra- or extracellular spaces. Recently, freeze etching techniques have been used in the study of the morphology and morphogenesis of a group A togavirus (21). These techniques have confirmed previous results obtained by workers who had used thin sectioning techniques and provided additional new information on the fine structure of the capsid and on the kinetics of the morphogenesis process. A further significant advance in our understanding of the site and mode of replication of both Group A and Group B togaviruses was made very recently. FRIEDMAN *et al.* (22) have succeeded in separating a unique cytoplasmic fraction from chicken embryo cells infected with Semliki Forest virus. This fraction was rich in special membranous structures, referred to in earlier studies as CPV-I.

This fraction was heavily enriched in pulse labelled RNA, viral RNA polymerase and viral RNA forms associated with RNA replication. Thus, electron microscopic findings were correlated with biochemical evidence that the isolated fraction contained a membrane-associated replication complex. QURESHI and TRENT (23) have also described the isolation of a replication complex from BHK cells infected with Saint Louis Encephalitis virus, a group B togavirus. This structure has an average sedimentation coefficient of 250, contains the viral RNA polymerase, various forms of viral RNA (42S, 26S and 20S) and virus-specific proteins.

IV. Molecular Structure and Composition of the Virions

Earlier information on the structure and composition of togaviruses was rather scarce. Group A viruses were visualized as spherical particles with a diameter of 40 to 60 nm. They were significantly larger than Group B, togaviruses (30 to 40 nm). The RNA content has been estimated as ranging from 4.4 to 8.7%. More precise molecular weight estimates of the A togavirus RNA's were reported to range from 3.4 to 4.5×10^6 daltons (24). Inactivation of viral infectivity by lipid solvents clearly indicated the presence of essential lipids in the virion and was generally accepted as evidence for the possession of an envelope by the virus particle (25). PFEFFERKORN and HUNTER (26) have shown that the lipid content of Sindbis virus is about 26% of the dry weight. These are mainly phospholipids and cholesterol. During recent years great progress has been made in the elucidation of the structure, composition and molecular properties of the togavirion. It has been demonstrated that certain members of Group A togaviruses—Sindbis and Semliki Forest—can be adequately purified and their components separated and isolated in pure form. Essentially, these viruses are composed of two major subviral constituents: a ribonucleoprotein core and a lipoprotein envelope (27-31). STRAUSS *et al.* (27, 28) have found that Sindbis virus contains two major proteins. The "core" protein has a molecular weight of about 30,000 and is complexed with a viral RNA molecule; this constitutes the core of the virus particle or nucleocapsid. The "membrane" protein has a molecular weight of ca. 53,000 and forms a complex with the viral lipids to form the envelope of the virus. Similar results were obtained from the analysis of the molecular structure and composition of Semliki Forest virus (29, 30). SIMONS and KÄÄRIÄINEN have shown that purified Semliki Forest virus can be separated into four major components: RNA, two peaks of protein, and lipids. One of the proteins with a molecular weight of 33,000-35,000 is associated with the viral RNA and the other (molecular weight ca. 50,000) constituted part of the viral envelope. Similar results for Semliki Forest virus were obtained by ACHESON and TAMM (30).

Amino acid analysis of these two proteins provided evidence that the core protein is relatively rich in lysine, a basic amino acid, whereas the envelope protein is rich in leucine and valine, two hydrophobic amino acids.

Further studies of Sindbis virus have shown that the envelope protein is a glycoprotein and contains 14% carbohydrates (32). The sugars include: glucosamine, mannose, galactose, fucose, and sialic acid. Digestion of the membrane glycoprotein with pronase, yields three types of glycopeptides (33). Their molecular weights are: 3200, 2800 and 1800 daltons. The smallest glycopeptide is estimated to contain nine sugar residues. There is adequate evidence that the carbohydrate portion of the envelope glycoprotein is specified by the host whereas the protein moiety is coded by the viral genome (34-35). COMPANS has recently shown that the glycoprotein of Sindbis virus can be completely removed from the virus particle by means of bromelain, without destroying the integrity of the viral membrane (36a). This would indicate that the glycoprotein is located on the outside of the virion. Actually, the spikes which cover the surface of the virion are glycoprotein in nature.

To all this accumulated information on the structure and composition of the Group A togavirions, have recently been added X-Ray diffraction data on Sindbis virus (36b). These investigations have shed additional light on the lipid and protein organization of the virion. They can be summarized as follows: the Sindbis virion is built of three principal structural domains: the core, the lipid and the outer protein. The outer radius of the core is about 205 Å. There are approximately 400 protein subunits of molecular weight 30,000 in the core. Its surface makes direct contact with the inner polar groups of the lipid which surrounds the core. The lipid is organized in a bilayer which completely surrounds the core. The "viral membrane" is composed of the lipid layer and a single type of glycoprotein of molecular weight estimated at 53,000 daltons. The glycoprotein molecules can be completely removed without destroying the integrity of the viral membrane, indicating that the glycoprotein is located outside the lipid layer. There seems to be very little contact, if any, between the outer protein and the core. The biochemical composition is 62% protein, 26% lipid, 6% RNA and 7% carbohydrate. The RNA of the virus is a single-stranded molecule of 4×10^6 mol. wt. The number of glycoprotein molecules in the virus membrane is estimated at 700 (34). In a recent report evidence is presented that the membrane protein of Sindbis virus may not be a single glycoprotein but may be composed of two separable glycoproteins (37).

The information on the molecular structure and biochemical composition of the members of togavirus Group B has accumulated at a much slower pace. The findings by STOLLAR (38) that the virion of dengue virus type 2 is composed of three major polypeptides have recently been

confirmed for two other Group B togaviruses. SHAFIRO *et al.* (39) have shown that the "core" of Japanese Encephalitis virus contained one polypeptide with a molecular weight of 13,500 daltons. The other two proteins, with molecular weights of 8700 and 53,000 respectively, are present in the viral membrane. The larger polypeptide is probably a glycoprotein. Similar though slightly different results were obtained upon analysis of the structural polypeptides of Saint Louis Encephatitis virus (40).

V. Immunological "Functions" of Subviral Components of Sindbis and of Eastern Equine and Western Equine Encephalitis Viruses

The use of detergents, such as sodium dodecyl sulphate, urea and reducing agents for the dissociation of the lipid containing togavirions has been practiced widely in most of the studies reported, for the analysis of virus structure and composition. These treatments caused the destruction of most of their biological activities. APPLEYARD *et al.* (41) have recently used less drastic methods for the dissociation of purified Semliki Forest virions. The most innocuous was found to be treatment with the non-ionic detergent, NP-40. They have demonstrated that, though viral infectivity is severely affected, other biological activities such as the complement fixing, haemagglutinating and neutralizing antibody blocking activities, can be fairly well preserved and their properties characterized.

We have also developed procedures for the purification of the Sindbis virion and for the separation and characterization of its major subviral constituents (7, 31). Detailed procedures, materials and method were reported previously (7, 31). The results of our studies on the structure and composition of Sindbis virus can be summarized, as follows (31): acrylamide gel electrophoresis of Sindbis virus nucleocapsids obtained from infected chick embryo cells and labeled with radioactive amino acids, showed the presence of only one protein species. A small quantity of a second protein, with a higher molecular weight, was also found. Electrophoresis of the purified mature Sindbis virions demostrated two proteins: the protein which was present in the "subvirions" and a second protein which was incorporated into the virions during the maturation process. This latter protein thus constitutes the protein component of the Sindbis virion envelope. This was demonstrated by electrophoresis in acrylamide gel of Sindbis virus labeled with ^{3}H-leucine and ^{14}C-glucosamine. The two viral proteins were demonstrated by the ^{3}H-leucine label. In addition, the slower migrating protein was also labeled with ^{14}C-glucosamine. This clearly indicated that the viral envelope is of a glycoprotein nature. These findings are in accord with those previously reported for Sindbis virus (27, 28, 32).

The nonionic detergent, Nonidet P-40, was used to separate the ribonucleoprotein core from the viral envelope. Following treatment of

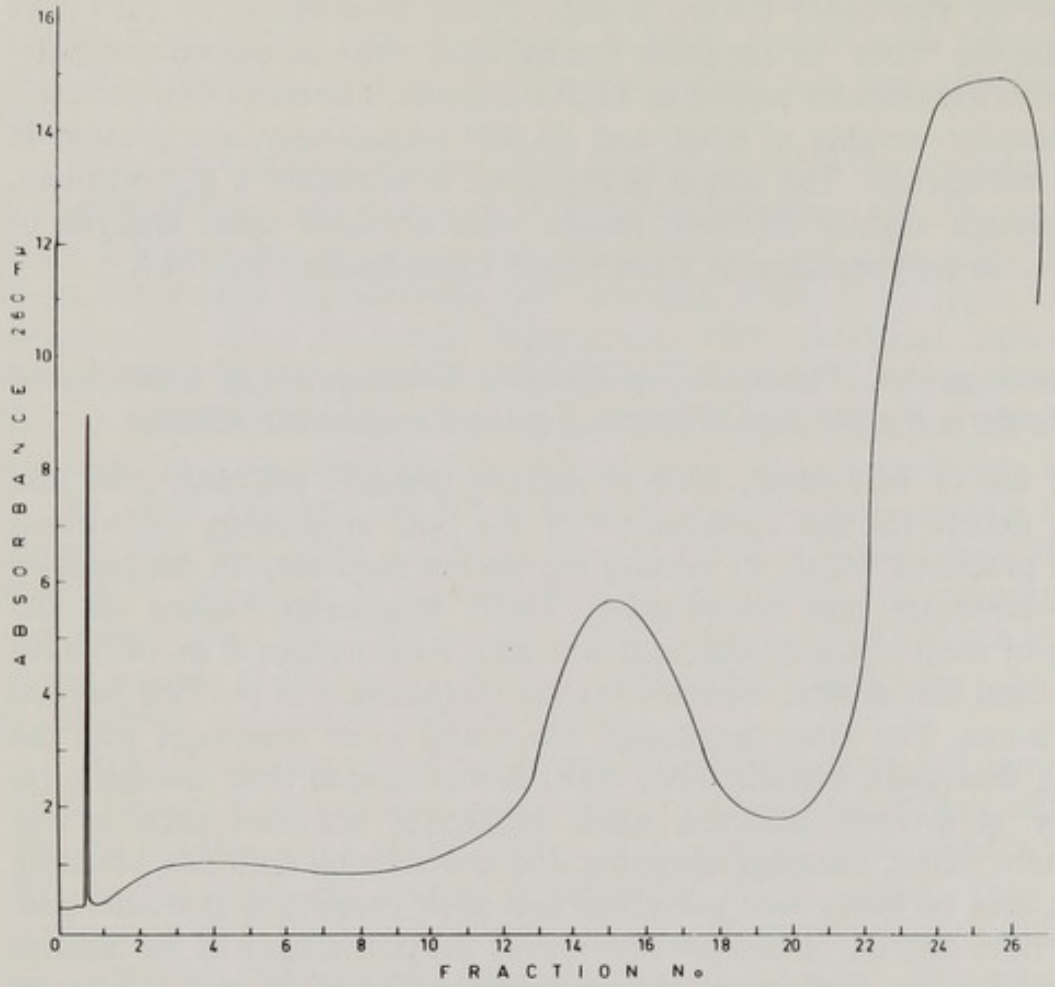

FIG. 1. Separation of purified EEE treated with Nonidet P-40 into the ribonucleoprotein core (fraction 15) and lipoprotein envelope (top of gradient).

purified Sindbis virus overnight at 4 °C with a 0.1% solution of Nonidet P-40, the suspension was banded in a sucrose gradient (15-30 w/w). Treatment with Nonidet P-40 resulted in the separation of Sindbis virions into a ribonucleoprotein core and a viral envelope. This technique turned out to be very convenient for the separation of the two viral components, the ribonucleoprotein (RNP) core and lipoprotein (LP) envelope, and was subsequently used for the separation of the subviral components of Eastern Equine Encephalitis (EEE) and Western Equine Encephalitis (WEE) viruses and in all immunological tests and procedures. Occasionally, the RNP core was re-banded in sucrose with essentially similar results.

Some of the results obtained were presented recently (42, 43). Figures 1 to 4 present some data on the structure and composition of EEE and WEE viruses. Figure 1 demonstrates the separation of purified virions of EEE virus into the ribonucleoprotein core and the viral envelope by the use of the nonionic detergent NP-40, following banding in a sucrose

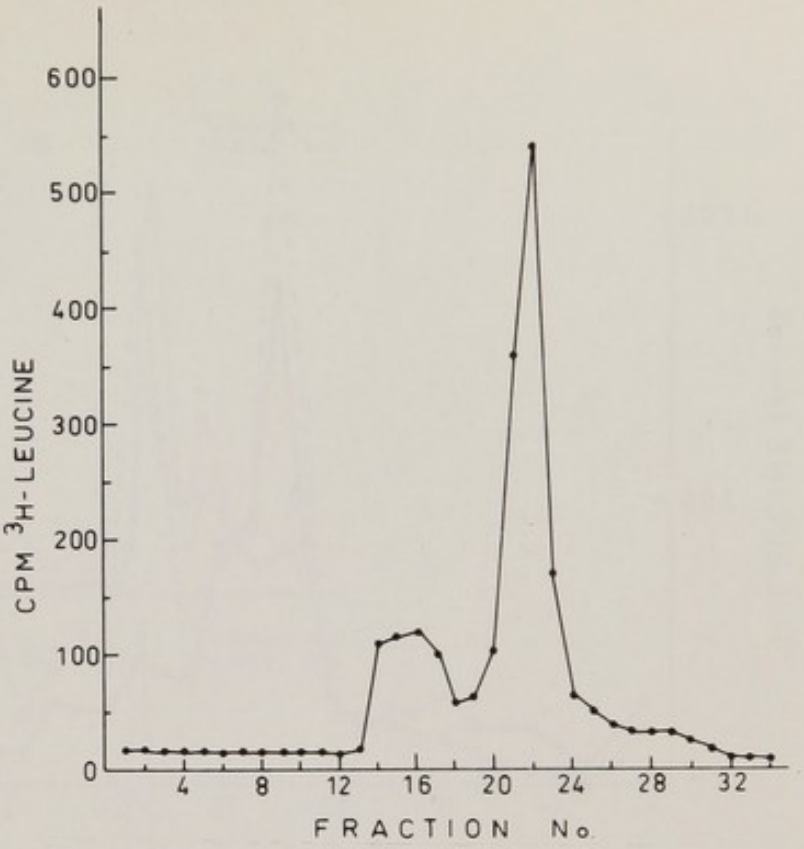

FIG. 2. Acrylamide gel electrophoresis of the separated ribonucleoprotein core of WEE.

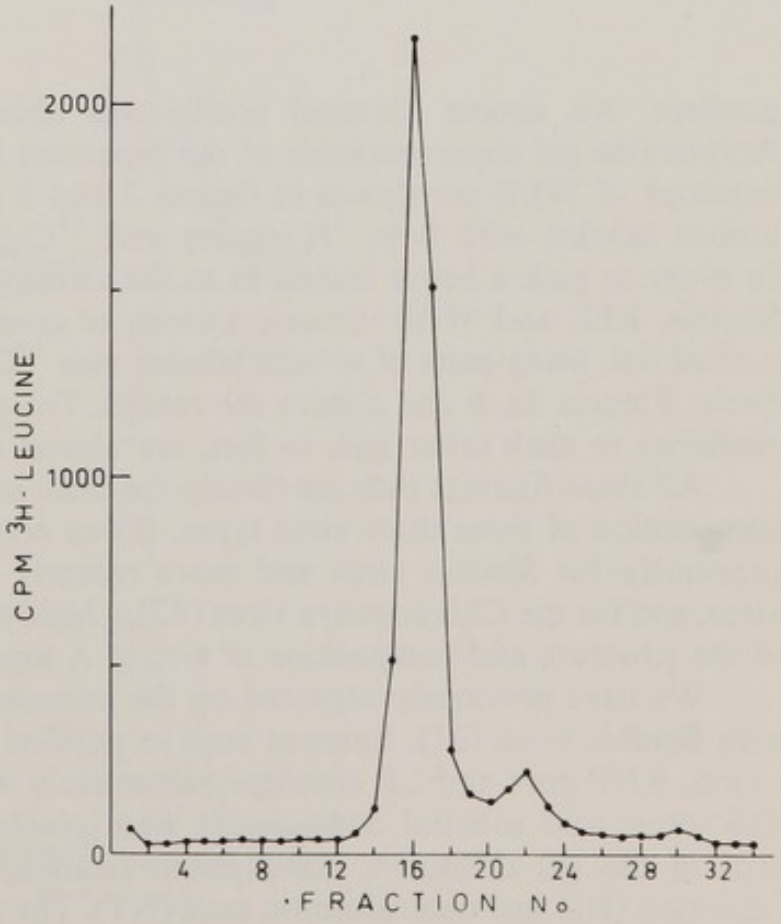

FIG. 3. Acrylamide gel electrophoresis of the separated envelope of WEE.

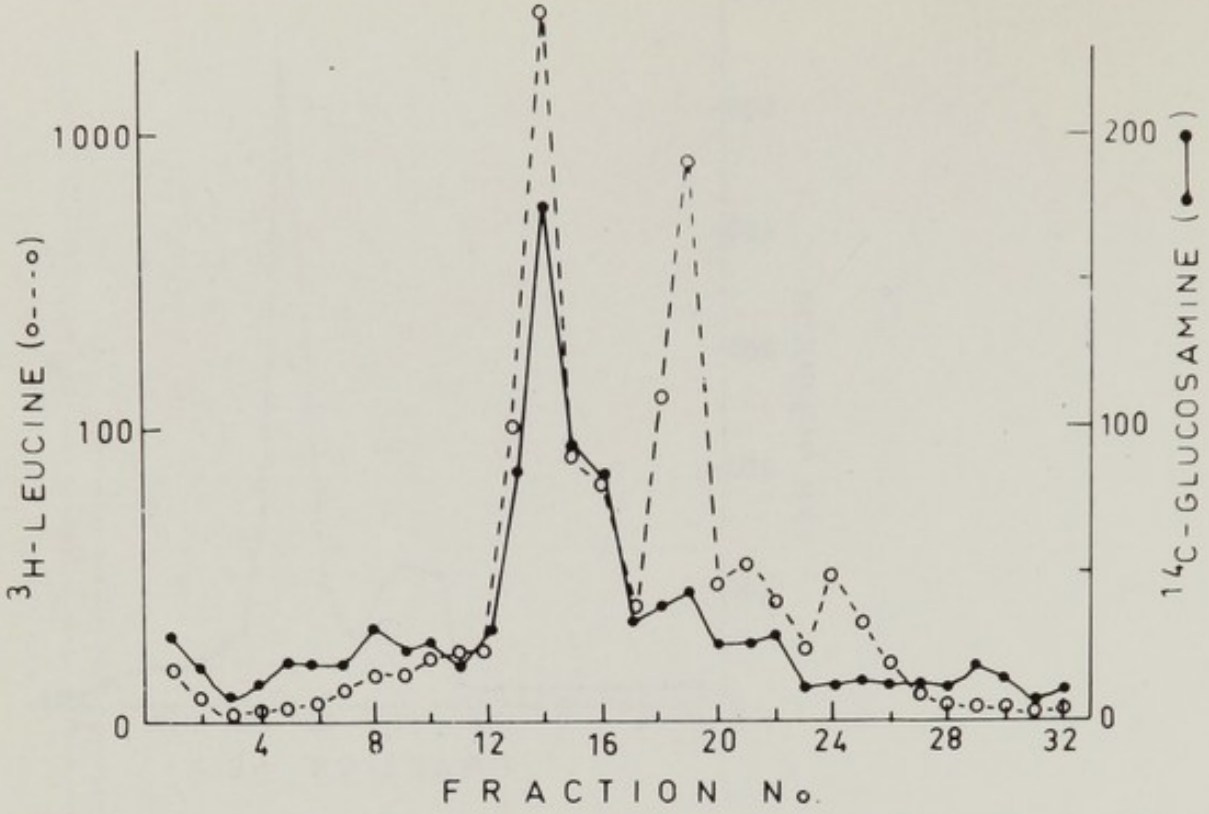

FIG. 4. Acrylamide gel electrophoresis of purified WEE labeled with ^{3}H-leucine and ^{14}C-glucosamine.

gradient. An almost identical profile was obtained for WEE virions. Acrylamide gel electrophoresis of the separated RNP core and the viral envelope of WEE are shown in figures 2 and 3 respectively, and that of virions labeled with both ^{3}H-leucine and ^{14}C-glucosamine in figure 4. In order to gain a better insight as to the identity of the structures of the Sindbis, EEE and WEE viruses, a series of co-electrophoresis runs was carried out, using pairs of viruses labeled with ^{14}C-and ^{3}H-leucine respectively. Figures 5a, b and c show the results. The profiles exhibit a striking similarity to each other and, in fact, are almost identical.

All these findings indicate clearly the close similarity in structure and composition of these three virus types. If one considers the data reported previously for Sindbis virus and more recently (41) for Semliki Forest virus, and for the Chikungunya virus (42), a highly uniform picture emerges of the structure and composition of Group A togaviruses.

We have previously reported on the immunological results obtained with Sindbis virus (31). Immune sera to purified preparations of Sindbis virion, RNP core and LP envelope respectively were prepared in rabbits. The virion and subviral components were cross-tested with the immune sera in the gel diffisuion, complement fixation, (CF) hemagglutination inhibition (HI) and neutralization tests (NT). The results of these serologic tests were very clear-cut. They demonstrated the presence of at least

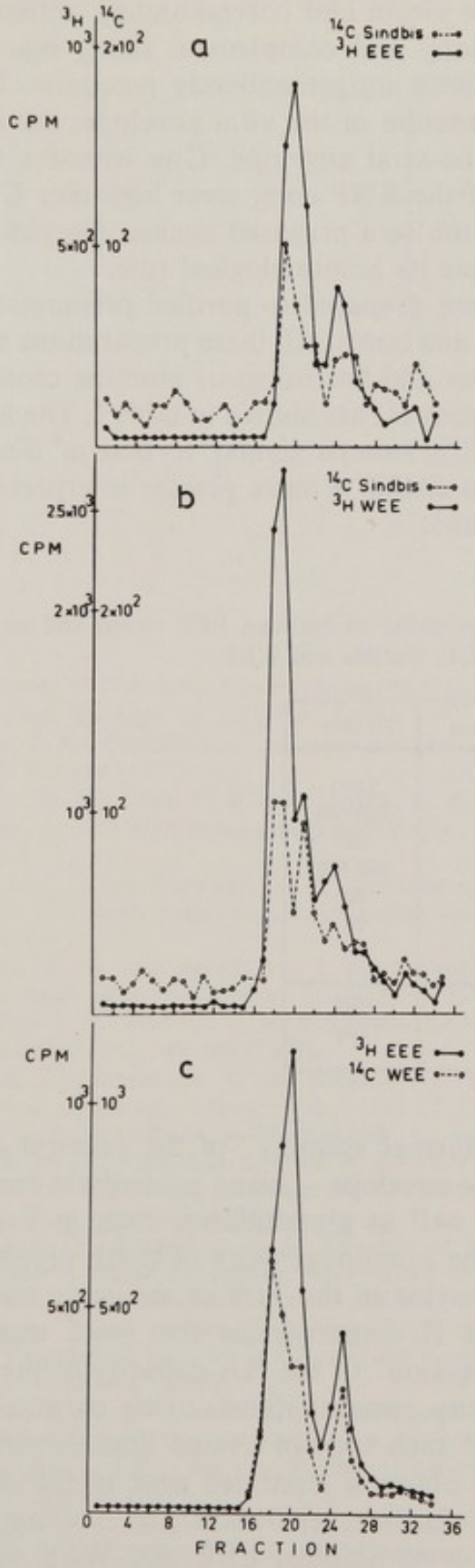

FIG. 5. Acrylamide gel co-electrophoresis of "pairs" of purified viruses: Sindbis, EEE, and WEE.

three distinct antigens in the virion and corresponding antibodies in the immune sera. The Ouchterlony and complement fixing reactions show distinctly the specificity of these antigen-antibody reactions. The hemagglutination is undoubtly a function of the viral envelope; the HI and NT antibodies are induced by the viral envelope. One wonders what is the immunological "function" of the RNP core, since high titer CF antibody against it is present in immune sera prepared against the virion. Further studies are needed to elucidate its immunological role.

Rabbit immune sera were prepared to purified preparations of EEE and WEE virions, envelopes and cores. All these preparations are presently being tested in homologous and heterologous immune cross-reactions. Results of a representative cross HI are shown in table I. The homologous HI reactions of EEE exhibit a pattern similar to that of Sindbis. More extensive tests are needed to enable a more precise interpretation of the heterologous immune reactions.

Table I. Cross hemagglutination-inhibition between EEE virion and immune sera to virion and viral components of EEE, Sindbis and WEE.

HA antigen	Immune serum against	HI titer
EEE virion	EEE virion	1280
	EEE envelope	320-640
	EEE core	20
	Sindbis virion	160-320
	Sindbis envelope	40
	Sindbis core	20
	WEE virion	20
Sindbis virion	Sindbis virion	2560
	EEE virion	160

We have carried out "further splitting" of the subviral components —the core and especially the envelope— using proteolytic enzymes, such as pronase and trypsin, as well as glycosidases, such as neuraminidase and β-galactosidase (44). The immunogenicity of these preparations and their direct and indirect behavior in the various serologic reactions is at present under study. Table II demonstrates that mild treatment with trypsin results in the "inactivation" of the HA capacity of the viral membrane whereas the CF activity remains intact; using chromatography of DEAE cellulose columns of such trypsin-treated Sindbis virus envelope preparations, we are able to obtain a separated peak of CF activity. This technique will be used for further separation and for molecular and immunologic characterization of Sindbis, EEE and WEE virus components.

Table II. Treatment of envelope preparations of Sindbis virus with proteolytic enzymes.

Enzyme[1]	Concentration	Titer	
		HA	CF
None	–	1024	128
Pronase	1 µg/ml	4	32
	10 µg/ml	4	32
Trypsin	1 µg/ml	64	256
	5 µg/ml	32	"
	50 µg/ml	8	"
	200 µg/ml	4-8	"
Bromelain	1.3 mgm/ml	8	256

[1]Treatment was carried out for 30 min. at 37 °C.

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REOVIRUS: A VIRUS WITH A SEGMENTED DOUBLE-STRANDED RNA GENOME

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CONTENTS

Reovirus: a virus with segmented double-stranded RNA genome

I. Introduction

There are some thirteen major classes of animal viruses, and each presents certain unique features. In addition, each possesses structural or biological attributes that, while not unique, are nevertheless best studied with it. All mammalian viruses thus deserve to be studied not only for their own sake, but also for the insight that their study provides into the nature of other viruses. Reovirus has been particularly profitable as a model virus, and it is therefore very appropriate that in this Symposium, in which we honor FRANCISCO DURÁN-REYNALS and which is devoted to the discussion of "Viral Replication and Cancer", we focus in some detail on the virus concerning the molecular biology of which more is known than that of most others.

I intend to focus on three specific aspects. First, I would like to discuss the nature of the components of reovirions and the manner in which they are arranged in the virus particle, since it is impossible to understand either viral replication or the effects that viruses have on their host cells without a clear understanding of what exactly constitutes a virion. Second, I will discuss the nature of reovirus uncoating, or rather the lack of it, since reovirus is unique among viruses in not having its genome physically separated from its protein coat. Third, I would like to examine how viral protein and RNA are synthesized in infected cells and to speculate on certain aspects of reovirus morphogenesis.

II. The Nature of Reovirions and Their Components

A. RNA

Reovirions contain two distinct types of RNA. About three-quarters of it is double-stranded (GOMATOS and TAMM, 1963) and represents the

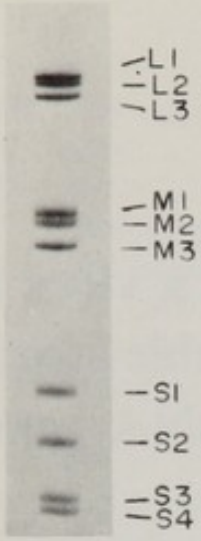

FIG. 1. The 10 segments of reovirus double-stranded genome RNA. Autoradiogram of a polyacrylamide gel. Electrophoresis was from top to bottom. (Courtesy of Dr. A. SCHÜRCH).

genome RNA. Its unique feature is that it exists not in the form of a single molecule, but as a collection of discrete and unique segments (BELLAMY *et al.*, 1967; WATANABE and GRAHAM, 1967), which can be readily separated by polyacrylamide gel electrophoresis (SHATKIN *et al.*, 1968). There are 10 such segments which fall into three size classes designated L, M, and S[1] (fig. 1), the approximate molecular weights of which are 2.7×10^6, $1{,}4 \times 10^6$ and $0.6\text{-}0.8 \times 10^6$ (BELLAMY *et al.*, 1967), corresponding to about 4500, 2300 and 1100 nucleotide base pairs. The 5'-terminal and 3'-terminal nucleic acid bases of all strands are G and C, respectively (BANERJEE and SHATKIN, 1971; BANERJEE *et al.*, 1971), suggesting that they are perfect duplexes.

The nature of the arrangement of the RNA segments within virions is not known. It has been inferred that they may be linked by very weak noncovalent bonds, because, when released gently, strands up to 7 μ long have occasionally been seen (DUNNEBACKE and KLEINSCHMIDT, 1967; GRANBOULAN and NIVELEAU, 1967). However, such strands, which are very rare, may well have arisen fortuitously; in addition, there is good evidence that all strands can be transcribed simultaneously by a polymerase present in the virion (see below). The strands may therefore not be linked at all. The primary reason for considering the possibility that the 10 genome RNA segments may be linked was that this would provide a mechanism for ensuring that each virion received one, and one only,

[1] Double-stranded RNA segments are designated by capitals (L, M and S); the corresponding single-stranded RNA species are designated by lower case letters (l, m and s) (BELLAMY and JOKLIK, 1967a).

segment of each species. As we shall see below, it is now clear that RNA segment assortment does *not* proceed at the level of double-stranded RNA segments; the raison d'être for a double-stranded RNA recognition and linkage mechanism has therefore disappeared. The important point about the reovirus genome is that it is segmented, and that it was the first animal virus genome for which this was rigorously shown to be the case. Now, of course, it is known that the orthomyxovirus genome is also segmented, as is that of RNA tumor viruses.

In addition to double-stranded RNA, reoviruses also contain considerable amounts of single-stranded RNA of low molecular weight. Since it is rich in adenine, it was first known as A-rich RNA (BELLAMY and JOKLIK, 1967b; SHATKIN and SIPE, 1968a). It is now known that this RNA is not homogenous, but that it consists essentially of two classes of molecules (NICHOLS *et al.*, 1972a) (table I). First, there is a series of molecules in which the only nucleic acid base is adenine; they range in length from 2 to about 20 nucleotides, and they have either PPP or PP or P (in decreasing order) at their 5'-termini. Their formula can thus be represented by $(p)(p)p(A)_{1\text{-}19}A_{OH}$; there are about 850 of such molecules present in each virion. They are now known as the reovirus *oligoadenylates*. Second, there is a series of oligonucleotides $(p)ppGC_{OH}$, $(p)ppGCU_{OH}$, $(p)ppGCUA_{OH}$, $(p)ppGCUA(A)_{1\text{-}2}A_{OH}$ and $(p)ppGCUA(U)_{1\text{-}4}U_{OH}$. These are the *5'-G-terminated oligonucleotides,* of which there are about 2,000 molecules per virion. In addition, there are about 350 other molecules from 2-8 nucleotides long (about 10% of the total), the sequence of which has not yet been determined.

It is clear that since these oligonucleotides have 5'-PPP groups, they do not represent random breakdown products of larger molecules, but rather they are initiated and transcribed by an RNA polymerase, presum-

Table I. The oligonucleotides present in reovirus particles.

Oligonucleotide(s)	Sequence	Chain length	Approximate number of molecules per virion
Oligoadenylates	$(p)(p)p(A)_{1\text{-}19}A_{OH}$	2-20	850
	$(p)ppGC_{OH}$	2	50
	$(p)ppGCU_{OH}$	3	900
5'-G-Terminated oligonucleotides	$(p)(p)pGCUA_{OH}$	4	775
	$(p)ppGCU(A)_{1\text{-}3}A_{OH}$	5-7	130
	$(p)ppGCUA(U)_{1\text{-}4}U_{OH}$	6-9	130
	Unknown	2-8	350

(From NICHOLS *et al.*, 1972a).

ably by that which is present in reovirus cores (see below). They represent, in all likelihood, products of abortive transcription by the core polymerase (see below). There are two arguments in favor of this hypothesis: immature reovirus particles which contain the full complement of double-stranded RNA but no oligonucleotides have been found in infected cells (ZWEERINK, personal communication); and the base sequence of the 5'-G-terminated oligonucleotides resembles the 5'-terminal sequence of one of the s species of messenger RNA, which is (p)ppGCCAUUUUUUGCU-(C,U)UCCAGACGUUG- (NICHOLS *et al.*, 1972b). As for the oligo-adenylates, a possible mechanism for their synthesis is reiterative copying by a slippage mechanism (CHAMBERLAIN and BERG, 1964) of sequences of uridylate residues such as occur in the sequence shown above. Neither class of oligonucleotide is essential for infectivity or for the expression of any other viral function.

B. Protein

Reovirions are composed of seven species of polypeptides which, like the double-stranded RNA species, fall into three size classes (fig. 2). Their molecular weights, percentage frequency and number of molecules of each per virion are listed in table II (SMITH *et al.*, 1969). The most striking feature about their sizes is the fact that they correspond to the coding capacities of several of the genome RNA segments: thus the λ, μ

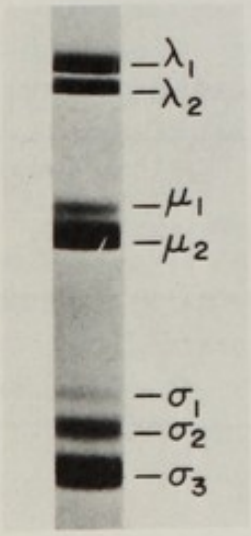

FIG. 2. The capsid polypeptides of reovirus. 7.5% polyacrylamide gel-0.1% SDS-6 M urea. Coomassie Brilliant Blue stain. Direction of electrophoresis from top to bottom.

Table II. Summary of reovirus capsid polypeptides.

Species	M.W.	Percent in virion	Approximate number of molecules per virion	Location	
λ1	155,000	15	107	core	
λ2	140,000	11	87	core	
μ1	80,000	2	23	core	
μ2	72,000	36	550		outer shell
σ1	42,000	1	31		outer shell
σ2	38,000	7	200	core	
σ3	34,000	28	900		outer shell

(From SMITH *et al.*, 1969).

and σ polypeptides comprise 1400-1500, 700-800, and 350-400 amino acids, while the L, M and S genome RNA segments consists of about 4500, 2300 and 1000-1300 nucleotide base pairs, respectively. This relationship suggests that the capsid polypeptides are coded by messenger RNAs transcribed from entire genome RNA segments. Strong evidence for this view has recently been obtained by the demonstration that each segment of genome RNA is indeed transcribed into a single-stranded RNA strand of exactly the same length, as far as one can tell, and that six of the single-stranded RNA species are translated by *in vitro* protein synthesizing systems into polypeptides of exactly the same size, as far as one can tell, as capsid polypeptides. The only exception concerns polypeptide μ2 which is not a primary gene product, but is derived by cleavage from a precursor, μ1, which is itself present in virions in small amounts. The evidence for this will be presented below.

Techniques have been devised for the isolation in quantities up to 10-100 mg of the major capsid polypeptide species λ1 and λ2, μ2, σ2 and σ3, utilizing column gel filtration procedures (PETT *et al.*, 1973). This has permitted analysis of their overall amino acid composition, investigation of their amino- and carboxyl-terminal amino acid sequences and the examination of their tryptic peptide maps. Their amino acid composition presents no unusual features. All reovirus capsid polypeptides except μ2 possess blocked amino-terminal amino groups, the nature of which are not yet known. Polypeptide μ2 does have a free amino-terminal amino group; its amino-terminal amino acid sequence is H_2N-Pro-Gly-Gly-Val-Pro-. This implies that when polypeptide μ1 is cleaved to μ2, it is the amino-terminal portion of the molecule which is removed. The carboxyl-terminal amino acids of several of these polypeptides have also

been determined by sequential degration with carboxypeptidase A and B. They are: polypeptide $\sigma 3$, -(val,val,leu)-COOH; $\mu 2$, -leu-(arg,tyr,tyr)-Arg-COOH; and either one or both of $\lambda 1$ and $\lambda 2$ terminate(s) in -Arg-COOH, with a different adjacent amino acid sequence from that of $\mu 2$ (PETT *et al.*, 1973). These data demonstrate that all the major reovirus capsid polypeptides are unique molecular species, and it is very likely that this is also true for the minor species $\sigma 1$.

C. Morphology

Reovirions possess a double-shelled capsid (GOMATOS *et al.*, 1962; JORDAN and MAYOR, 1962; LOH *et al.*, 1965), but the precise structure of neither the outer nor the inner shell has so far been established with certainty. The reason for this is that although both are obviously composed of capsomers, it has so far not been possible to visualize them clearly enough to determine their spatial interrelationships. It has been postulated that the outer shell consists either of 92 hollow prismatic capsomers arranged on the surface of an icosahedron (VÁSQUEZ and TOURNIER, 1962; MAYOR *et al.*, 1965), or of 180 solid capsomers positioned equidistantly from the center of the virion and around 92 holes (VÁSQUEZ and TOURNIER, 1964; AMANO *et al.*, 1971). Either of these arrangements would require the presence of 18 peripheral capsomers.

Recent work has shown that this model may have to be revised. While, as pointed out above, reovirus capsomers are too indistinct in head-on aspect to permit one to deduce how they are arranged, they can be visualized clearly at the particle periphery: there are 20, not 18 (LUFTIG *et al.*, 1972) (fig. 3). Furthermore, if there are 20 peripheral capsomers,

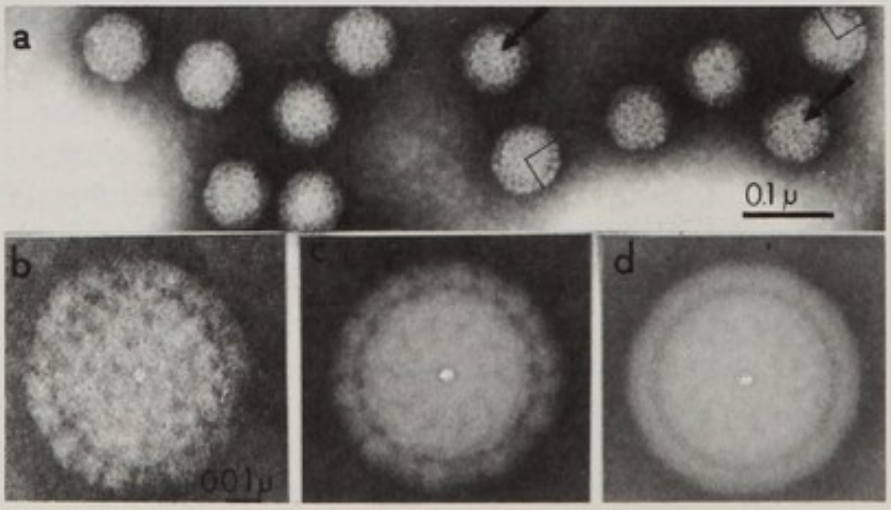

FIG. 3. (a) Intact reovirus stained with 4% PTA. × 135,000. Arrows indicate capsomers in head-on aspect. (b), (c), and (d) represent $\eta = 0$, 10 and 12-fold rotations, respectively, for a selected particle. × 450,000. (From LUFTIG *et al.*, 1972).

exactly on a meridian, and slightly fewer, if, as is likely, this is not the case. In any case, the number differs significantly from either 92 or 180 as required by the two models mentioned above.

The outer shell of reovirions is readily digested by chymotrypsin (SHATKIN and SIPE, 1968b; SMITH *et al.*, 1969). The structure which remains is the *core;* it consists of the inner capsid shell which still contains the nucleic acid in nuclease-resistant form. The most obvious morphological features of cores are 12 projections or spikes, 9.5 nm in diameter with a central channel 4.7 nm wide, which are arranged as if on the 12 five-fold vertices of an icosadeltahedron (LUFTIG *et al.*, 1972) (fig. 4 and 5). Cores also exhibit capsomers, which are smaller than those of the outer shell (4 nm diameter as against 9 nm). They are also too indistinct to be assigned to any particular geometric arrangement, but once again they can be visualized clearly at the periphery, and again there are 20, not 18. In summary, the outer and inner shells of reovirions appear to be constituted according to similar if not identical symmetry principles; the icosahedral distribution of the spikes indicates that these are icosahedral symmetry principles; and the presence of 20 peripheral capsomers suggests that the total number of capsomers in each shell is of the order of 120-127. The class of icosahedral deltahedra which satisfies all these requirements best is that for which P= 3, T= 12, and the number of morphological subunits is 122.

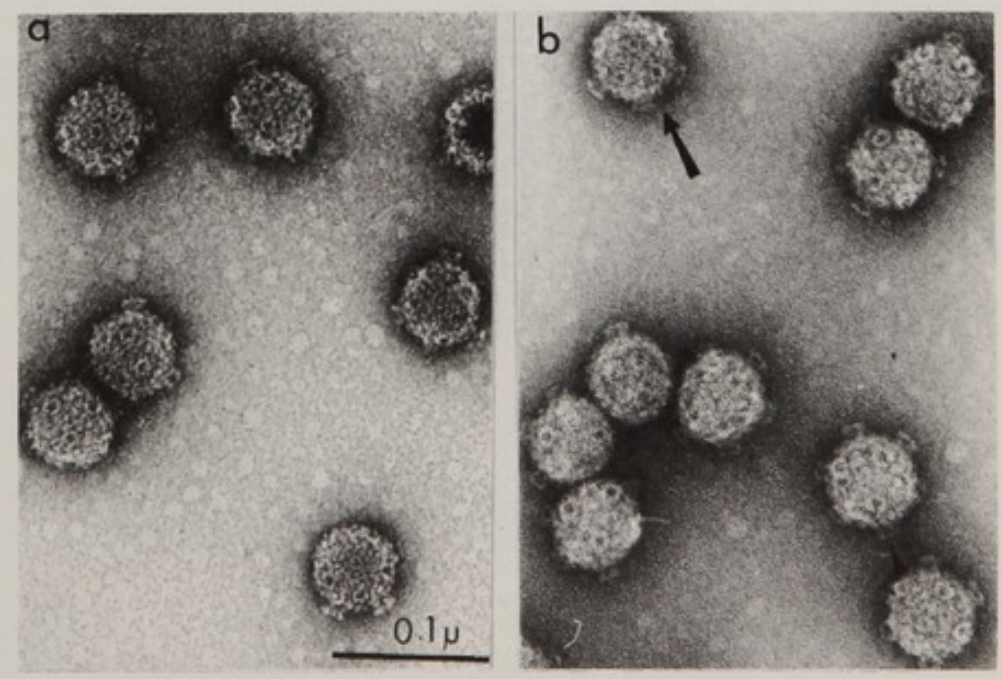

FIG. 4. Reovirus cores fixed with glutaraldehyde and stained with 2% uranyl acetate exhibit surface projections or spikes (arrow). Their central, stain-permeable channel is indicated by the arrowhead. ×225,000. (From LUFTIG *et al.*, 1972).

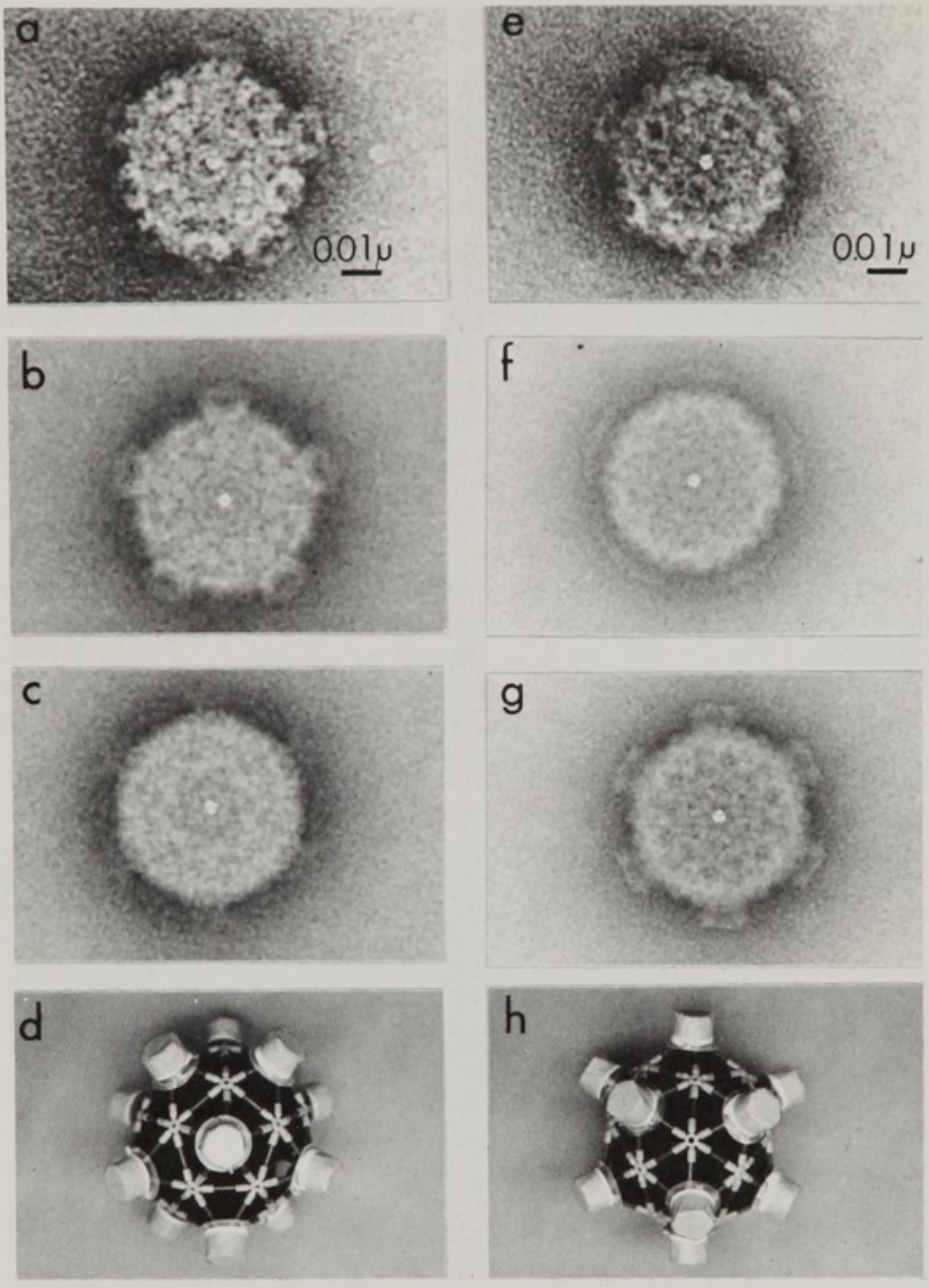

FIG. 5. The Markham rotation technique was applied to two reovirus core particles whose central axis was either on a presumptive 5-fold (a) or 3-fold vertex (e). Enhancement of the five peripheral spikes of (a) was achieved by an $\eta = 5$ (b), but not an $\eta = 6$ rotation (c). A model was constructed to depict the spike orientation when the central axis is through a 5-fold vertex (d). Enhancement of the six peripheral spikes of (e) was exhibited with an $\eta = 6$ (g), but not an $\eta = 5$ rotation (f). A model with the central axis through a 3-fold vertex is shown in (h). Staining of (a) and (e) as described in fig. 4. ×450,000. (From LUFTIG *et al.*, 1972).

D. The Topography of the Capsid Polypeptides

Having defined the morphology of reovirions, we may now examine how the seven capsid polypeptides are arranged within them. The diameter of the reovirion is 76.5 nm, that of the core 52 nm, and that of the cavity within the inner shell 42 nm (LUFTIG *et al.*, 1972). This means that the core corresponds to about 32% of the virion's volume and the central cavity, where the nucleic acid is located, to about 16.5% of that volume. The polypeptide composition of the outer shell is readily deduced from the relative polyacrylamide gel electrophoretic patterns of dissociated virions and cores: it comprises polypeptides $\sigma 3$, $\mu 2$ and $\sigma 1$, which together make up about 65% of the total virion polypeptides (SMITH *et al.*, 1969) (fig. 6, panels A and G). Digestion of virions with very low concentrations of chymotrypsin shows that these three polypeptides are removed sequentially in a highly reproducible and characteristic manner (JOKLIK, 1972): polypeptide $\sigma 3$ is removed first, followed by $\mu 2$, and finally by $\sigma 1$ (fig. 6). The resultant cores are very much less infectious than virions (about 10^{-5}); infectivity is lost when $\mu 2$ begins to be degraded. Both $\sigma 3$ and $\mu 2$ are hydrolyzed via a series of readily identifiable intermediate fragments which remain transiently associated with the virus particles for some time (fig. 6). Interestingly enough, the nature of the first bonds of $\mu 2$ to be cleaved can be influenced experimentally (JOKLIK, 1972).

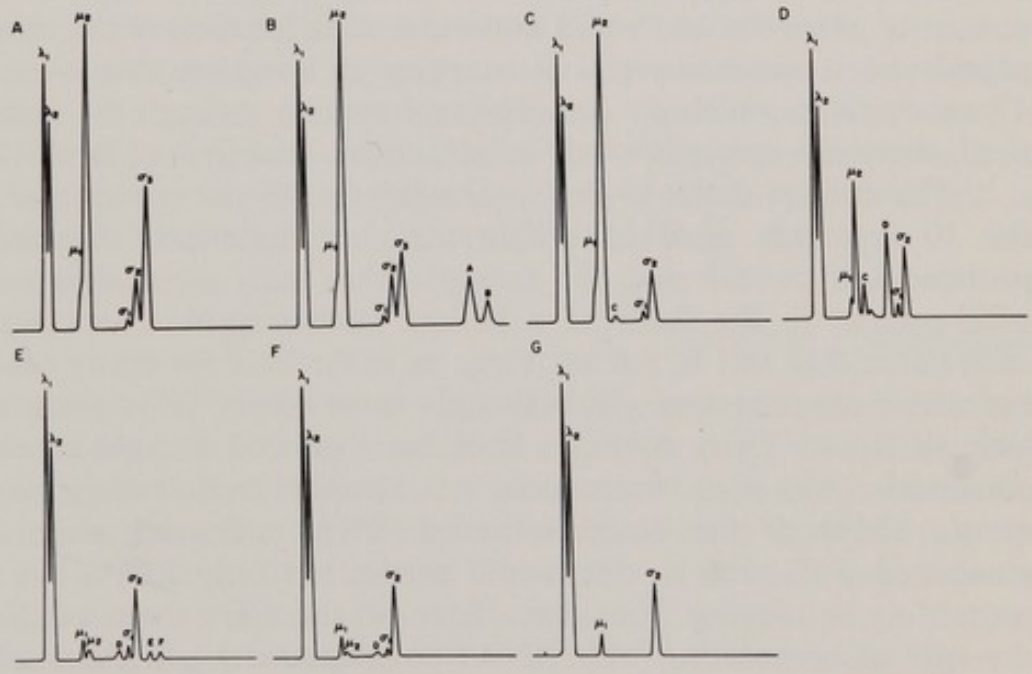

FIG. 6. Polyacrylamide gel electrophoresis profiles of polypeptide components of reovirions treated with chymotrypsin (10 μg/ml) for various periods of time. (A) 0 min; (B) 5 min; (C) 14 min; (D) 18 min; (E) 22 min; and (F) 30 min. The profile in panel (G) represents the end product of limit digestion (60 min with 1000 μg/ml enzyme) and corresponds to that of typical reovirus cores. Polypeptides A, B, C, D, E and F are the fragments which remain transiently associated with the virus particles (see text). (From JOKLIK, 1972).

At low concentrations of chymotrypsin, polypeptide $\mu 2$ (M.W. 72,000) is split to yield a polypeptide with a molecular weight of 64,000 which is then further degraded; but at high enzyme concentrations a polypeptide with a molecular weight of 60,000 appears, which is not further degraded, nor is polypeptide $\sigma 1$ then removed. As a result, digestion of reovirions, especially those of the Carter strain of reovirus type 3, with high concentrations of chymotrypsin does not yield cores, but particles which lack polypeptide $\sigma 3$ and a 12,000 dalton fragment of $\mu 2$. These particles are fully infectious, and have been designated *paravirions* (JOKLIK, 1972). Similar particles have been found independently by SHATKIN and LAFIANDRA (1972). By contrast, the particles lacking polypeptide $\sigma 3$ and the 8000 dalton fragment of polypeptide $\mu 2$ which are formed during the normal course of digestion at low concentrations of chymotrypsin are *not* infectious.

Although we know a good deal about the morphology of the outer reovirus shell and its polypeptides, we cannot yet define the former in terms of the latter. No free capsomers have yet been isolated either as a result of breaking down virions or from infected cells. Brief treatment with chymotrypsin such as would be expected to remove polypeptide $\sigma 3$ renders the outlines of the capsomers sharper, although still not sharp enough for their spacial interrelationships to be determined (LUFTIG *et al.*, 1972); this may indicate that $\sigma 3$ is not a component of capsomers, but that it is located between them. If this were true, capsomers would consist of either six or five $\mu 2$ molecules. The location of the minor polypeptide $\sigma 1$ is not known; it is tempting to speculate that overlying the 12 core spikes, which do not penetrate entirely through the outer capsid shell, there are specially modified structures, and that $\sigma 1$ is part of them.

The core presents several problems. One is the question of whether the 10 segments of RNA within them are intimately associated with protein; it is conceivable, for example, that each segment is complexed with protein in the form of a helical nucleocapsid. There are several indications that this is not so. First, as is the case for many other icosahedral viruses, reovirus yields include some empty virus particles which lack all RNA. These particles have been isolated and their polypeptide constitution has been determined: it is identical to that of virions (SMITH *et al.*, 1969). If the double-stranded RNA molecules were normally associated with protein, one would expect not only RNA, but also this protein to be missing from them. Second, there are three candidates for the role of complexing with RNA: polypeptides $\lambda 1$, $\lambda 2$ and $\sigma 2$. (Polypeptide $\mu 1$ can scarcely be considered, since there are only about 20 molecules of it in each virion, that is, only about two per RNA molecule). Polypeptides $\lambda 1$ and $\lambda 2$ could not fulfill this role since each amounts to about 13% of the virion's mass, and the cavity available is only about 16% of the reovirion's volume: there would not be enough space to permit

all molecules of either of these polypeptides to be complexed with RNA. Polypeptide $\sigma 2$ only comprises 7% of the total virion polypeptide and could conceivably be combined with the nucleic acid; but not only would there still be a space problem, and the problem of why it is still present in undiminished amounts in empty particles, but the ratio of protein: RNA in the complex would only be about 0.5, not nearly enough for a structure even remotely resembling the sort of nucleocapsid present in myxoviruses or rhabdoviruses. The most reasonable conclusion is that the genome segments of reovirions exist within the central cavity as naked double helices.

The second problem concerns the location of the various polypeptides in the actual structure of the core which clearly has two components: the shell proper and the spikes. The only piece of evidence available so far is that only two of the four core polypeptides can be iodinated and are therefore presumably the only ones which are located on the outer surface of the core (although it is conceivable that polypeptides which present no groups which can be iodinated are also located there): these are polypeptides $\lambda 2$ and $\mu 1$ (MARTIN and ZWEERINK, 1973; see also LEWANDOWSKI and TRAYNOR, 1972). Now, the ratio of $\lambda 2$ to $\mu 1$ (in terms of mass) is about 10:1; and the ratio of shell proper to spikes (in terms of surface area) is also about 10:1. It is conceivable therefore that $\lambda 2$ occupies the outer surface of the shell proper, and that $\mu 1$ is located at the outer surface of the 12 spikes. Since the skipes account for 15-20% of the core's mass and therefore about 5-7% of the virion's mass, $\mu 1$ cannot be the only component of spikes; conceivably spikes are composed of polypeptide $\mu 1$ together with polypeptide $\sigma 2$, which together amount to about 8-9% of the virion's mass. If this is correct, then the inner core surface would be occupied by polypeptide $\lambda 1$. Much further work will be necessary to determine whether this model is correct.

E. The Core Transcriptase

The double-stranded genome segments of reovirus cannot themselves serve as messenger RNA. Such messenger RNA is provided by the action of a transcriptase which appears to be part of the reovirus core. The enzyme is inactive while the outer shell is complete or even after polypeptide $\sigma 3$ has been removed; but once polypeptide $\mu 2$ begins to be cleaved (1) virus particles become clumped; (2) infectivity is abolished (see above); (3) oligonucleotides leak out and (4) transcriptase becomes activated. The fact that all these changes occur at precisely the same stage of disassembly of the outer capsid shell suggests that they are the result of the same molecular rearrangement (JOKLIK, 1972).

The reaction catalyzed by the transcriptase is the completely asymmetric transcription of the 10 genome RNA segments into single-stranded

RNA molecules which possess the same polarity (designated plus) as reovirus messenger RNA isolated from polyribosomes of infected cells (HAY and JOKLIK, 1971). They are *complete* transcripts since (a) when hybridized to their corresponding minus strands they form hybrids with exactly the same electrophoretic mobilities as the ten double-stranded genome RNA segments which are present in virions (unpaired tails would slow them down); (b) they can be translated *in vitro* into 8 polypeptides, 6 of which have exactly the same electrophoretic migration rates as capsid polypeptides (GRAZIADEI and LENGYEL, 1972; MCDOWELL *et al.*, 1972); and (c) the 5'-terminal nucleotide base sequence of one of them (NICHOLS *et al.*, 1972b), which commences with (p)ppGCC-, is compatible which the finding that the 5'-termini of all double-stranded RNA molecules is ppGPy- (BANERJEE and SHATKIN, 1971).

The transcription catalyzed by the reovirus core transcriptase is fully conservative, that is, neither of the two template strands appears among the products (SHEKEL and JOKLIK, 1969) (fig. 7). Its action is therefore analogous to that of the classical DNA-dependent RNA polymerases; it is a DS→SS RNA polymerase. Under optimal conditions all ten template segments are transcribed at the same rate, that is, equal masses of all ten transcripts are formed (fig. 8); as a consequence, the number of molecules of the various transcripts formed is inversely proportional to their molecular weight (SHEKEL and JOKLIK, 1969). The rate of transcription is about 7-8 nucleotide residues per chain per second. This rate is fast enough for

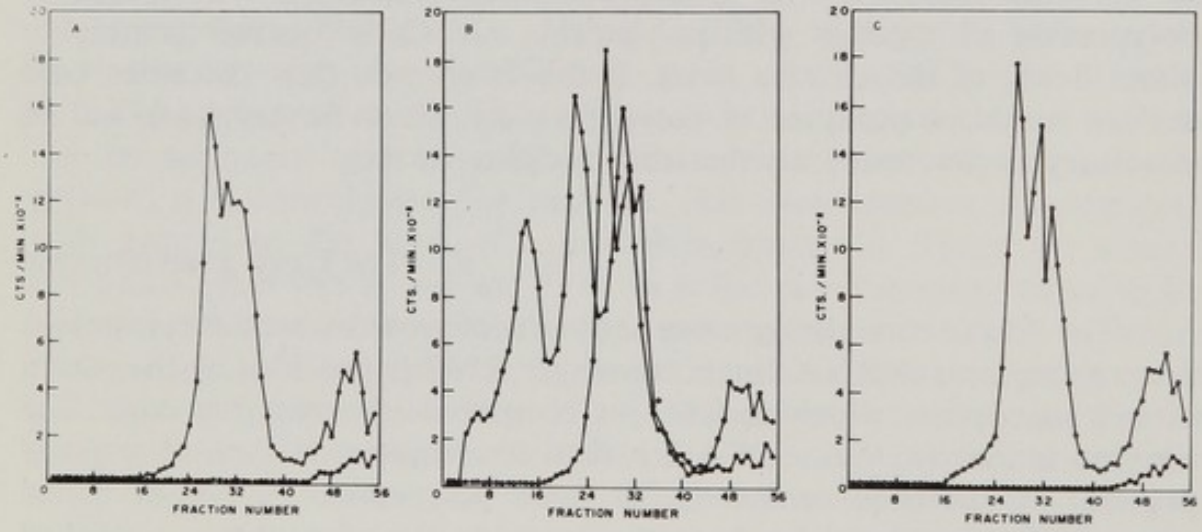

FIG. 7. Demonstration that reovirus transcription is conservative. (A) Reovirus cores containing ^{14}C-labeled RNA (open circles) were incubated for 3 hr at 37 °C with ^{3}H-UTP (closed circles) in the absence of ATP, GTP and CTP; all RNA was then solubilized and analyzed in sucrose density gradients (direction of sedimentation from right to left). The template was still intact; no transcripts were formed. (B) The reaction mixture included ATP, GTP and CTP. The three size classes of transcripts were synthesized; the template was still intact. (C) Exactly the same as B, except that the reaction mixture was treated with RNase prior to solubilization so as to hydrolyze the transcripts. The template was still intact. (From SKEHEL and JOKLIK, 1969).

the amount of RNA synthesized by even relatively small amounts of cores in brief periods of time to be measurable in terms of its optical density at 260 mμ (fig. 9). Since the enzyme is also very stable, large amounts of reovirus messenger RNA can readily be prepared.

A question of considerable interest concerns the number of transcriptase catalytic sites per core. This question has been answered most

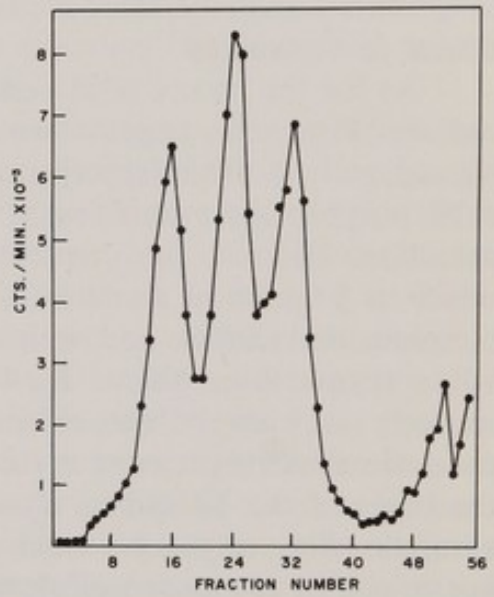

FIG. 8. Sucrose-SDS density gradient radioactivity profile of the transcriptase reaction products. The reaction mixture contained in 0.8 ml: 1.25 mg virus; 80 μg chymotrypsin; 400 μg Macaloid (previously incubated at 40° for 90 min); 1.2 mg each of ATP, GTP, CTP and UTP; UTP-^{3}H at a final specific activity of 1.63 μCi/mole; 12 μmoles $MgCl_2$; 80 μmoles Tris HCl, pH 8; 15 μmole PEP; and 50 μg pyruvate kinase. Incubation was for 40 min at 40°. The SDS-sucrose density gradient (15-30%) was centrifuged at 20° at 24,000 rpm for 20 hours in rotor SW27. (direction of centrifugation from right to left). (From SHEKEL and JOKLIK, 1969).

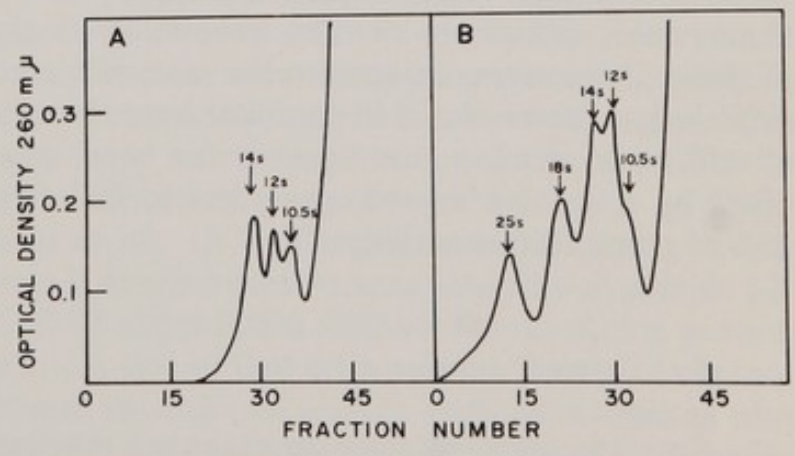

FIG. 9. Optical density profiles of transcriptase reaction products centrifuged in sucrose-SDS density gradients. Reaction mixture as for fig. 8 with radioactive nucleoside triphosphate omitted. Panel A, zero time reaction mixture (showing the profile of template RNA); Panel B, 40 min reaction mixture (showing the transcript profile superimposed on the template profile). (From SKEHEL and JOKLIK, 1969).

definitively by direct visual observation of cores in the process of transcribing RNA: cores from which up to nine individual strands of RNA are extruded, each apparently from a different source on the core, have been observed (GILLIES *et al.*, 1971). This strongly suggests that there is at least one catalytic site for each genome segment and that all ten segments can be transcribed simultaneously; further, it is very tempting to speculate that the transcripts are released through the channels in the center of the spikes.

As for the nature of the enzyme, there seem to be at least three alternatives. First, the enzyme may consist of polypeptides other than $\lambda 1$, $\lambda 2$, $\sigma 2$ and $\mu 1$. This is very unlikely, as the presence of as many as ten such polypeptide molecules per virion is very unlikely to have escaped detection. Second, the enzyme might be polypeptide $\mu 1$, a polypeptide which is present in small numbers (approximately twice the number of template molecules), and which could move readily along the templates while transcribing them. However, this alternative is also considered unlikely in view of the evidence presented above. Third, the enzyme catalytic sites might exist on the inside of the core shell,[2] possibly near the bases of the 12 spikes, close to the putative release channels; and the catalytic sites might be located not merely on one polypeptide chain, but might be composed of elements of several species of polypeptides. In this connection it has already been pointed out (JOKLIK, 1970) that the three major components of reovirus cores ($\lambda 1$, $\lambda 2$ and $\sigma 2$) bear a remarkable resemblance to the three polypeptides (β', β, and α) which comprise *E. coli* DNA-dependent RNA polymerase, both with respect to size and relative amount. The fact that a change in molecular configuration activates the enzyme (see above) would be explicable on the basis that in virions the catalytic site would not exist, but that incipient hydrolysis of polypeptide $\mu 2$ would bring the appropriate portions of polypeptide chains into apposition, thereby generating catalytic sites. Proof of one of these alternatives, or some other one, will come only when the transcriptase has been isolated in core-free form. In spite of strenuous attempts, all efforts to achieve this have so far been unsuccessful; disruption of cores by a wide variety of agents has so far always resulted in complete loss of transcriptase activity.

F. Other Enzymes

Reovirus cores catalyze an exchange reaction between the four ribonucleoside triphosphates and inorganic pyrophosphate (WACHSMAN

[2] There are no catalytic sites on the *outside* of the core shell, since cores are unable to transcribe exogenous double-stranded RNA.

et al., 1970). The only single triphosphate to support this exchange is GTP, with which the rate is about one-half of that with all four. The fact that the optimal conditions for pyrophosphate exchange are similar to those for the transcriptase, and that individually only GTP, which is known to be present at the 5'-termini of reovirus RNA strands, supports it, suggests that the exchange reaction represents part of the overall transcriptase reaction; however, the degree of participation of individual nucleoside triphosphates in the exchange reaction is not related to the overall base composition of reovirus RNA.

Reovirus cores also possess a nucleoside triphosphate phosphohydrolase activity which liberates inorganic phosphate from all common ribo- and deoxyribonucleoside triphosphates (KAPULER *et al.,* 1970; BORSA *et al.,* 1970). Apparently the same catalytic site is responsible for all four activities, the relative rates of hydrolysis being ATP > GTP > CTP > UTP. The enzyme differs in its response to metal cations and in the nature of the mutual competitive inhibitions among nucleoside triphosphates from all other known nucleoside triphosphate hydrolases (BORSA *et al.,* 1970). Its function, and the advantge its presence confers on reovirions are not clear. Presumably it is responsible for converting some of the terminal 5'-triphosphate groups of both genome RNA and its transcripts to diphosphates (BANERJEE and SHATKIN, 1971; BANERJEE *et al.,* 1971; NICHOLS *et al.,* 1972b).

III. The Uncoating of Reovirus

Like many other viruses, reovirions are phagocytosed by cells, and then concentrated in lysosomes in which part of their outer shell is removed, and from which they are subsequently released into the cytoplasm (SILVERSTEIN and DALES, 1967; SILVERSTEIN *et al.,* 1972). However, in distinction to all other viruses so far investigated, the reovirus genome is not liberated, that is, it is not separated physically from its capsid. This has been shown as follows. Very soon after infection the buoyant density of parental virus changes from its normal value of 1.36 g/cc in CsCl to 1.38 g/cc (SILVERSTEIN *et al.,* 1970; CHANG and ZWEERINK, 1971). The reason for this change is that parental virions lose all polypeptide $\mu 3$, as well as a fragment of $\mu 2$ of about 8,000 daltons; the remaining portion of $\mu 2$, which is very similar in size to that arising in the course of chymotryptic degradation of virions *in vitro* (see above), remains associated with the resulting subviral particles and no further change in their polypeptide composition then occurs. Just as in the case of the reaction *in vitro,* loss of $\sigma 3$ and the $\mu 2$ fragment results in the activation of the transcriptase (LEVIN *et al.,* 1970), and transcripts which possess mRNA activity are released into the cytoplasm, where they are translated. The parental

genomes are not liberated; the subviral particles remain perfectly stable within the infected cell, and no free parental RNA can be detected in the cytoplasm (CHANG and ZWEERINK, 1971). During the later stages of the infection cycle the density of the subviral particles returns to a value only slightly in excess of that of virions (SILVERSTEIN *et al.*, 1970; CHANG and ZWEERINK, 1971); the reason for this is that newly synthesized polypeptide $\sigma 3$ attaches to them, thus generating particles identical to virions except that they do not contain polypeptide $\mu 2$, but only a 64,000 dalton fragment of it. This addition of $\sigma 3$ occurs efficiently in cell-free extracts, which has permitted the demonstration that it abolishes transcriptase activity (ASTELL *et al.*, 1972). It has not yet been determined whether the reconstituted virion-like particles are infectious.

Reovirus is thus unique among viruses in that its genome is not uncoated. Genetic information is passed on from parent to progeny solely via plus-stranded transcripts synthesized by the transcriptase in subviral particles, which have the dual function of serving as templates for polypeptide synthesis and for the synthesis of RNA strands with opposite (minus) polarity (see below), thereby generating progeny double-stranded genomes. We will now discuss each of these two functions in turn.

IV. The Synthesis of Reovirus-Coded Polypeptides

A. *In Vivo* Studies

It was pointed out above that six of the capsid polypeptides of reovirus correspond in size exactly to polypeptides expected to be coded by six of the double-stranded genome segments. These are polypeptides $\lambda 1$ and $\lambda 2$, expected to be coded by two of the three segments L1, L2 and L3; $\mu 1$ expected to be coded by M2; and $\sigma 1$, $\sigma 2$, and $\sigma 3$, expected to be coded by S1, S2, and S4 (see below). The synthesis of most of these polypeptides can be readily discerned in infected cells by taking advantage of the fact that reovirus replication is not inhibited by concentrations of actinomycin D which inhibit ribosomal RNA synthesis completely and host mRNA synthesis very extensively. It is therefore possible to determine by means of polyacrylamide gel electrophoresis which virus-specified proteins are formed throughout most of the multiplication cycle (ZWEERINK and JOKLIK, 1970; ZWEERINK *et al.*, 1971). The following observations have been made:

(1) The three size classes, λ, μ and σ, of virus-specified polypeptides are synthesized as early as 1-2 hr after infection; their rate of synthesis reaches a maximum at about 4-8 hr after infection, depending on the multiplicity of infection and the temperature of incubation. The relative rates of synthesis of these three classes do not change with time; therefore,

there is no change in the nature of the controls governing virus-specified polypeptide synthesis during the infection cycle. Indeed, all reovirus capsid polypeptides may be regarded as early proteins, since all are synthesized at the nonpermissive temperature by temperature-sensitive mutants unable to synthesize progeny double-stranded RNA (Ito and Joklik, 1972; Fields *et al.*, 1972).

(2) Analysis by polyacrylamide gel electrophoresis followed by autoradiography indicates that the various virus-specified polypeptides which are formed in infected cells fall into several categories (Zweerink *et al.*, 1971):

(a) Capsid polypeptides which are primary gene products. These are $\lambda 1$ and $\lambda 2$, $\mu 1$, and $\sigma 1$, $\sigma 2$, and $\sigma 3$. With the exception of $\mu 1$, they are all synthesized in amounts which correspond approximately to the frequency with which they occur in virions. All become labeled without a lag, indicating that they arise directly, rather than by cleavage of a precursor (fig. 10).

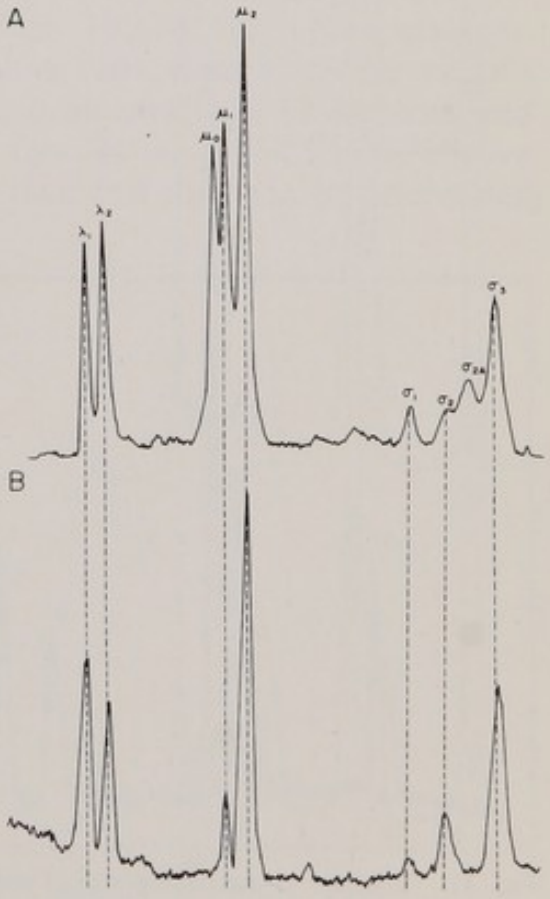

Fig. 10. Densitometer tracings of autoradiograms of cells infected for 17.5 hr at 31 °C and then labeled with valine-^{14}C (2 μCi/ml) for 120 min (A); and of reovirus capsid polypeptides derived from purified reovirions (B). Ten percent polyacrylamide gels were used; electrophoresis was carried out at 4 mA/gel for 60 hr from left to right. (From Zweerink *et al.*, 1971).

(b) Capsid polypeptides which are not primary gene products. Capsid polypeptides $\mu 2$ does *not* become labeled immediately; its amount relative to that of the other capsid polypeptides increases as the duration of labeling is increased, indicating that it arises by cleavage of a precursor. This precursor appears to be polypeptide $\mu 1$, as can be shown by means of pulse-chase experiments in which the amount of label in $\mu 1$ decreases as that in $\mu 2$ increases (fig. 11).

(c) Noncapsid polypeptides. In addition to the 7 capsid polypeptides, two polypeptides are synthesized in infected cells which are also of a size consistent with their being coded by reovirus genome RNA segments. The first of these is a polypeptide slightly larger than $\mu 1$, which has been designated $\mu 0$ (figs. 10 and 11). It is formed in large amounts; in fact, it is among the two most abundantly synthesized reovirus-specified polypeptides. It appears to be a component of intermediates in reovirus

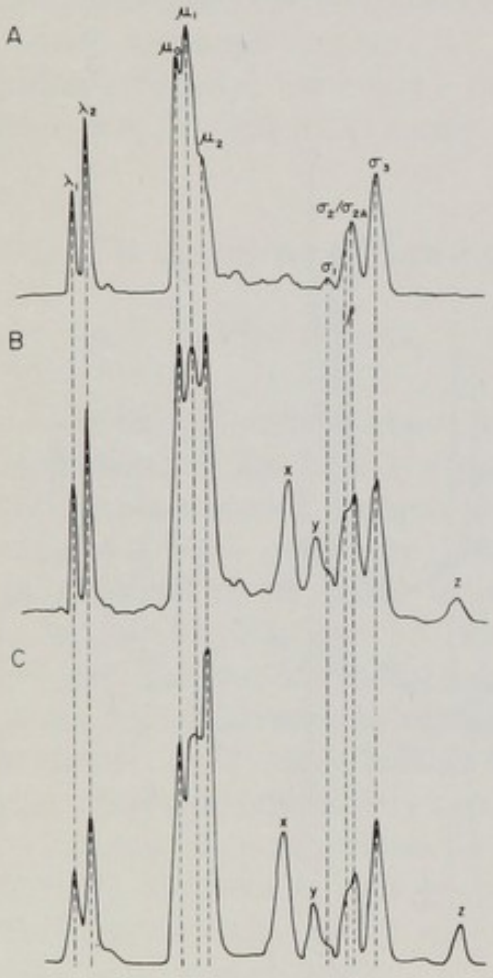

FIG. 11. Tracings of autoradiograms of polyacrylamide gels in which extracts of cells infected for 5 hr at 37° and labeled as follows had been electrophoresed: A, cells labeled for 20 min; B, cells labeled for 120 min; C, cells labeled for 20 min and then chased for 100 min. Electrophoresis was carried out in 7.5% gels at 5 mA/gel for 18 hr. The direction of electrophoresis was from left to right. The noncapsid nonessential polypeptides are x, y and z. (From ZWEERINK *et al.*, 1972).

morphogenesis (ZWEERINK, personal communication) (see below). The second is a polypeptide intermediate in size between $\sigma 2$ and $\sigma 3$; it has been designated $\sigma 2A$. Its function is not yet known.

(d) Noncapsid nonessential polypeptides. Several polypeptides are formed in infected cells at 37 °C which do not appear at 31 °C. These polypeptides are products of proteolytic cleavage of one or more of the capsid polypeptides, most probably $\mu 1$ or $\mu 2$ (fig. 11). They are very similar in size to the intermediates of *in vitro* chymotryptic cleavage of $\mu 2$ which were discussed above. They appear mostly when cytophatic effects are marked, particularly at temperatures of 37 °C and above, but not when infection proceeds at lower temperatures. It is conceivable that cytophatic damage causes lysosomes to become unstable, and that proteolytic enzymes leaking out of them degrade those capsid polypeptides which are most sensitive to them, namely those of the outer shell. Since these polypeptides are not formed at 31 °C, when reovirus multiplies to considerably higher titers than at 37 °C, they are obviously not essential to viral development, and they are therefore classed as nonessential.

(3) Some reovirus-specified polypeptides are synthesized in amounts greatly exceeding those of others (see figs. 10 and 11). These amounts, however, bear little relation to the relative rates with which the various species of viral mRNA are transcribed in infected cells (ZWEERINK and JOKLIK, 1970) (fig. 12; compare with figs. 10 and 11). These relative rates remain the same from the earliest time that they can be determined

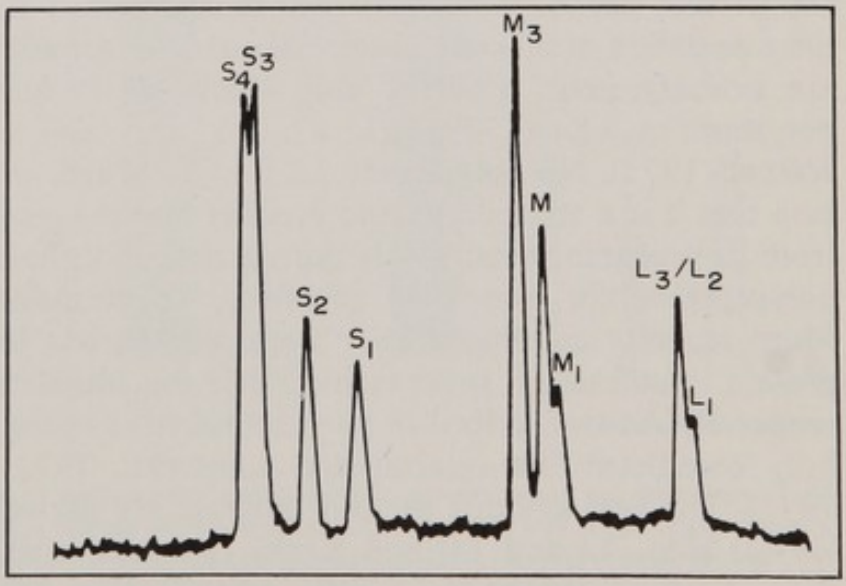

FIG. 12. Relative rates of formation of reovirus messenger RNA species during the period from 6 to 8 hr after infection. The profile represents the microdensitometer tracing of a gel autoradiogram of labeled mRNA hybridized with unlabeled double-stranded RNA. Electrophoresis was from right to left. The relative rates of formation of the various mRNA species were very similar during the periods from 2-4, 3-5 and 4-6 hr after infection. (From ZWEERINK and JOKLIK, 1970).

accurately, that is, at about 2 hr after infection, to the end of the multiplication cycle. Yet the structures from which reovirus mRNA is transcribed at early and late stages of the multiplication cycle are very different: during the early stages mRNA is transcribed from subviral particles derived from parental virions, while at late stages it is transcribed from progeny immature particles (see below). The amount of mRNA transcribed from the latter probably exceeds that transcribed from the former by a considerable margin.

It is not yet known why some mRNA species are translated far more frequently than others. Although it has been reported that all species of mRNA are present in polyribosomes in roughly equal amounts (WARD *et al.*, 1972), which suggests that all species of mRNA have roughly equal affinity for ribosomes, there is nevertheless little doubt that the reason for their widely differing translation efficiencies is to be sought in the nature of the nucleotide sequences at their 5'-termini. It is primarily in order to discover how the nucleotide sequences between the 5'-terminus and the initiation codon regulate frequency of translation that we are engaged in sequencing the various reovirus mRNA species (NICHOLS, HAY and JOKLIK, 1972, unpublished results).

B. *In Vitro* Studies

The preceding studies on the translation of reovirus mRNA in infected cells have been extended to *in vitro* systems. First, it has been shown that polyribosomes isolated from infected cells complete *in vitro* the translation of all eight species of reovirus-specified polypeptides which are primary gene products and which are formed *in vivo:* these are polypeptides $\lambda 1$, $\lambda 2$, P0, $\mu 1$, $\sigma 1$, $\sigma 2$, $\sigma 2A$ and $\sigma 3$ (MCDOWELL and JOKLIK, 1971). No polypeptide $\mu 2$ is synthesized, confirming the conclusion that it is a secondary gene product and suggesting that its formation from its precursor occurs only during virus morphogenesis. In this system polypeptides are completed faithfully, but translation is not initiated. More recently conditions have been worked out for preparing cell-free protein synthesizing systems in which the translation of reovirus messenger RNAs transcribed *in vitro* by reovirus cores is initiated and faithfully completed (GRAZIADEI and LENGYEL, 1972; MCDOWELL *et al.*, 1972). The best systems for this purpose are derived from rabbit reticulocytes (figs. 13 and 14) and Krebs II mouse ascites cells; but systems derived from L cells, HeLa cells, and Chinese hamster ovary cells are also able to accept added reovirus mRNA, and they also translate faithfully the majority of, if not all, reovirus mRNA species.

In all these systems, both *in vivo* and *in vitro*, the maximum number of primary gene products which can be detected is eight; yet there are

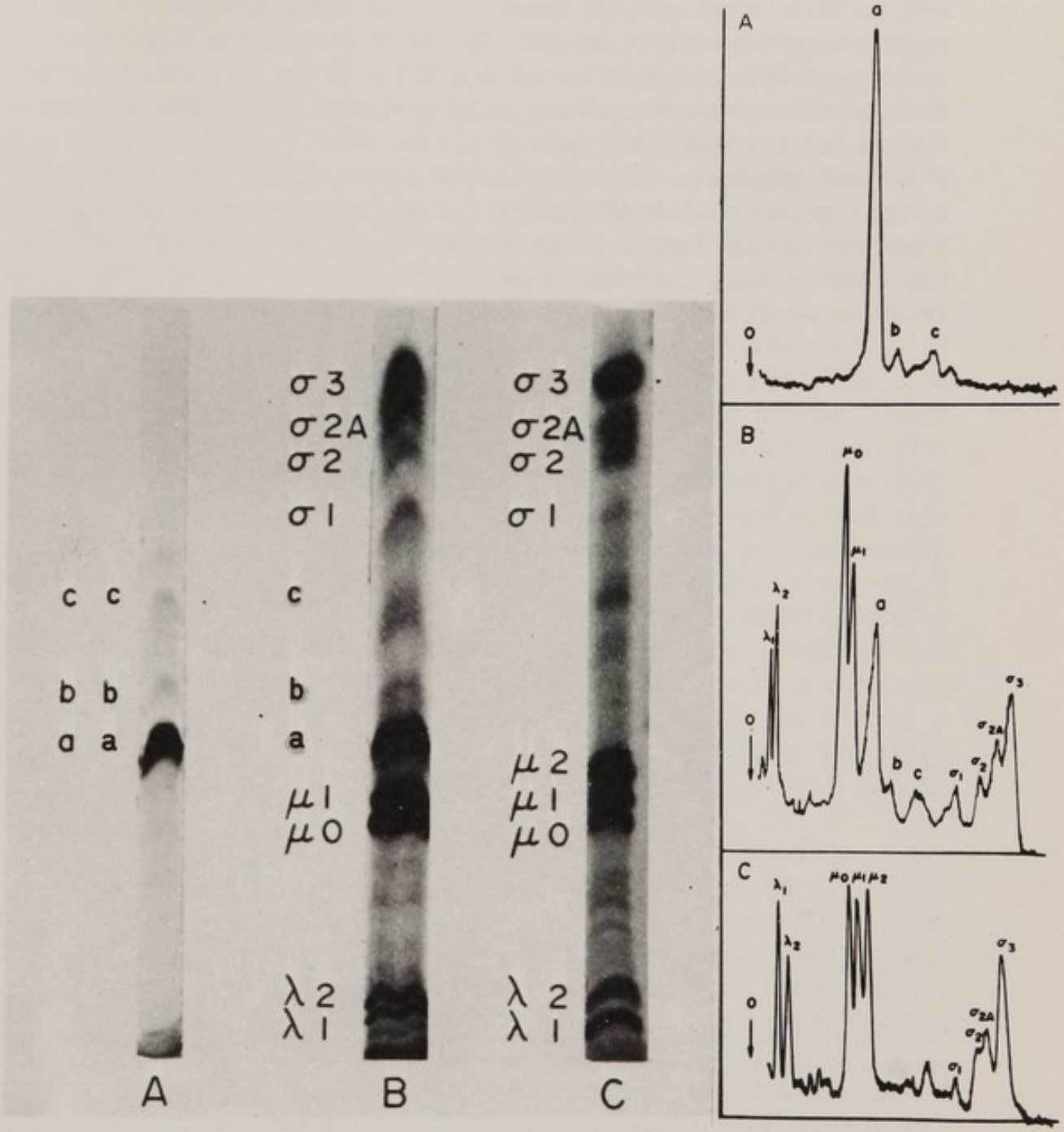

FIG. 13. Autoradiograms of polypeptides synthesized in a reticulocyte cell-free extract and separated by sodium dodecyl sulfate-gel electrophoresis. Migration was from bottom to top. (A) Polypeptides synthesized *in vitro* due to endogenous message; (B) polypeptides synthesized *in vitro* in the presence of 200 μg/ml of reovirus message; (C) viral polypeptides synthesized *in vivo*. (From McDOWELL *et al.*, 1972).

FIG. 14. Absorbance traces of the autoradiograms shown in fig. 13. Migration was from left to right. (A) Polypeptides synthesized *in vitro* due to endogenous message; (B) polypeptides synthesized *in vitro* in the presence of 200 μg/ml of reovirus message; (C) viral polypeptides synthesize *in vivo*. (From McDOWELL *et al.*, 1972).

10 species of mRNA. The relationship between the electrophoretic migration rates of reovirus genome RNA segments and virus-specified polypeptides, which permits identification of the polypeptides coded by each genome segment, is shown in fig. 15. It is clear that no polypeptide corresponding to either L2 or L3 and M3 have yet been found. Among the reasons why the two missing polypeptides have not yet been detected may be (a) that they are translated in very small amounts only; (b) that they coelectrophorese with other λ and μ polypeptides; and (c) that two of the reovirus mRNA species are not monocistronic but polycistronic. However, in that case also they would have to be translated only very infrequently, since no minor polypeptide species were detected in the *in vitro* systems, which are both sensitive and relatively free of background.

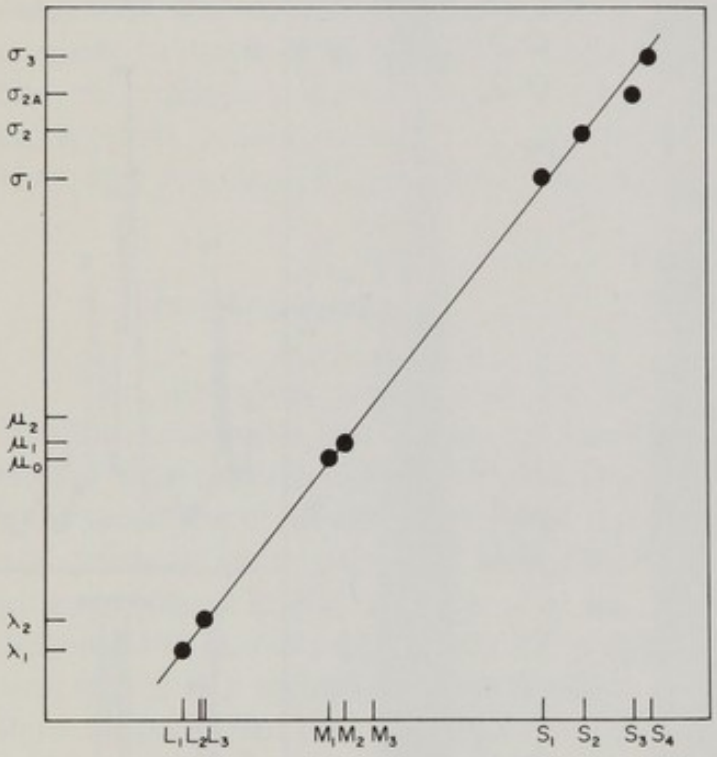

FIG. 15. Relative electrophoretic migration rates of reovirus genome RNA segments and reovirus-specified polypeptides. Distances are measured in arbitrary units. (From ZWEERINK *et al.*, 1971).

V. The Synthesis of Progeny Double-Stranded RNA and the Nature of the Structures in which it is Formed

Although the transcription of double-stranded RNA catalyzed by reovirus cores is formally analogous to the transcription of DNA by RNA polymerase in being conservative, the manner in which double-stranded reovirus RNA replicates bears no resemblance to DNA replication: DNA

replication is semiconservative; while double-stranded reovirus RNA replication is conservative. This became clear when it was found that parental reovirus RNA is not uncoated, but, apart from being transcribed into single-stranded RNA, remains inert within the parental subviral particles (SILVERSTEIN *et al.*, 1970; CHANG and ZWEERINK, 1971). Progeny double-stranded RNA molecules are formed in two stages, as first shown by SCHONBERG *et al.* (1971) who found that the plus and minus strands of progeny double-stranded RNA molecules are not formed simultaneously, but sequentially. This was demonstrated in the following manner. Assume that the two strands of progeny double-stranded RNA molecules are formed simultaneously; if label is present when they are formed, they will both be labeled. If these molecules are hybridized with a large excess of unlabeled plus strands, which can be easily prepared as the products of the *in vitro* transcriptase reaction (see above), then one-half of the label will appear in the hybridized double-stranded product; for the labeled minus strands will easily find plus-stranded partners, while the labeled plus strands will be diluted by the great excess of added unlabeled plus strands. If during the period of labeling only minus strands are synthesized, then *all* label will appear in the hybridized double-stranded product; if only plus strands are synthesized, then none of the label, or only a very small amount of it, depending on the extent of the excess of added unlabeled plus strands, will appear in the product. When this type of analysis was performed at various times during the reovirus multiplication cycle, the results obtained were consistent with the notion that the plus strands of progeny reovirus genome RNA were formed early during the cycle, while the minus strands were synthesized at later stages, when double-stranded RNA was actually being formed.

The synthesis of minus strands has recently been studied intensively in extracts of infected cells. The following information has been obtained:

I. The newly synthesized minus strands do not separate from their plus-stranded templates (SAKUMA and WATANABE, 1971); minus strand synthesis results in the formation of double-stranded RNA and is detected as such. The enzyme which catalyzes this reaction may be described as the SS→DS RNA polymerase.

II. Equivalent numbers of all species of double-stranded RNA are formed. Structures which contain intact plus strands and incomplete minus strands are intermediates in the formation of double-stranded RNA; and minus strand synthesis proceeds in the 5' to 3' direction (SAKUMA and WATANABE, 1972a).

III. Minus strand synthesis proceeds only for a brief period of time, which under optimal conditions does not exceed 10 min (ZWEERINK *et al.*, 1972). This suggests that plus strands are transcribed once and once only, and that no new structures in which minus strand synthesis can be initiated are formed in the *in vitro* system.

IV. The template for minus strand synthesis is destroyed by treatment with ribonuclease, demonstrating not only that it is accessible to the enzyme, but also that it is single-stranded (Acs *et al.*, 1971).

V. The structures within which double-stranded RNA is formed are normally associated with membranes, and when separated from them, sediment with 300-600S; their buoyant density in CsCl is 1.34 g/cc, 0.02 g/cc less than that of virions. Double-stranded RNA is not released from these structures but remains associated with them; when treated with chymotrypsin, they are converted to particles which have a buoyant density of 1.43 g/cc, the same as that of cores (Zweerink *et al.*, 1972; Sakuma and Watanabe, 1972b).

VI. Attempts to characterize the structures in which double-stranded RNA is formed are complicated by the fact that they seem to represent the first, or one of the first, stage(s) on the reovirus morphogenetic pathway which comprises numerous intermediates that are difficult to separate. Some of these intermediates possess DS→SS RNA polymerase activity and appear to be responsible for synthesizing a large part of the plus-stranded RNA which is formed in infected cells (see above).

The simplest model to account for all these results is the following. Infection starts with parental virions losing polypeptide $\sigma 3$ and a small portion of $\mu 2$, thereby being converted to subviral particles. These particles transcribe their genome RNA into plus-stranded RNA which has two functions: to act as templates for polypeptide synthesis and to act as templates for minus strand synthesis. The latter seems to proceed in structures consisting of one molecule of each of the ten plus-stranded RNA species and certain virus-specified polypeptides (including polypeptide $\mu 0$) (Zweerink, personal communication) and perhaps some host-specified polypeptides. Apparently the formation of these structures occurs predominantly in association with membranes, presumably so as to provide the appropriate three-dimensional environment, and may well involve highly specific polypeptide-RNA interactions. Within these structures the SS→DS RNA polymerase then transcribes the plus strands into minus strands which remain associated with them; simultaneously more capsid polypeptides are added; the DS→SS RNA polymerase generates more plus strands which mostly serve as templates for polypeptide synthesis in order to provide more capsid polypeptides; and the particles mature via a series of stages into the mature virions.

A. The Generation of Oligonucleotides

This brings us to the final point, namely the presence in reovirions of large numbers of oligonucleotide molecules. Within the cell these oligo-

nucleotides are not found free but only in association with particulate fractions; furthermore, they are formed at about the same time as double-stranded RNA (BELLAMY and JOKLIK, 1967b). Some of the immature virus particles described in the preceding section do *not* contain oligonucleotides, although they contain double-stranded RNA (ZWEERINK, personal communication); virions contain both. The question therefore arises as to at which stage of the maturation sequence they are formed. The explanation which we currently favor is as follows. As we have seen, the DS→SS RNA polymerase is inactive in mature virions; it is activated by removal of polypeptide $\sigma 3$ and a small portion of $\mu 2$, and is inactivated by addition of $\sigma 3$ (see above). Further, transcription of the double-stranded RNA segments is presumably brought about by the movement of RNA relative to the enzyme sites. It is conceivable that one of the last stages of maturation concerned with the addition of polypeptide $\sigma 3$ to the outer capsid shell results in conformational changes at the inner capsid surface which either prevents the motion of RNA relative to the enzyme, and/or distorts the catalytic site so that it becomes unable to function; in either case transcription would cease. It is conceivable that this cessation of activity does not occur instantaneously, but in stages, the first being immobilization, the second inactivation. However in the immobilized state the enzyme may still be able to catalyze nucleotide addition; but only very short stretches of template would presumably be transcribed. The striking similarity between the base sequences of some of the oligonucleotides and the nucleotide base sequence at the 5'-terminus of the one reovirus mRNA species which has been sequenced suggests that this may indeed occur. For a brief interval of time (say on the order of 15 sec) short stretches at the 5'-termini of double-stranded RNA segments may therefore be transcribed, but transcription would be very quickly aborted; and since at the same time the outer capsid shell would be completed, the transcripts would be sealed into virions. Thus the oligonucleotides would have no biological function and would not be necessary for infectivity (KRUG and GOMATOS, 1970; JOKLIK, 1972; SHATKIN and LAFIANDRA, 1972); rather they would represent byproducts of transcription encapsidated in virions as the result of an idiosyncracy of one of the final stages of reovirus morphogenesis.

VI. Conclusion

In summary, I have attempted to discuss current knowledge concerning the molecular biology of a well-characterized mammalian virus, reovirus, with particular reference to the nature of the virus-specified RNA species, both double-stranded, single-stranded and oligonucleotide, the nature of the capsid polypeptides, and the morphology of the virion;

the unique character of the reactions immediately following reovirus infection, which result not in the physical separation of the viral genome from its capsid, but in the generation of subviral particles capable of transcribing the double-stranded genome into plus-stranded RNA; the nature of the polypeptides for which the viral RNA codes, both *in vivo* and *in vitro;* and finally, the manner in which progeny double-stranded genomes are formed and the nature of immature reovirus particles. Information similar to that presented here will have to be provided for tumor viruses also. Lytic viruses such as reovirus provide very useful model systems for tumor viruses; and as we intensify our efforts to discover how tumor viruses interact with their host cells, we must be very careful not to abandon work on the lytic viruses.

VII. Acknowledgments

I would like to thank the numerous colleagues who collaborated with me in much of the work described in this paper. Among them were Drs. A. R. Bellamy, B. N. Fields, A. J. Hay, Y. Ito, S. S. Kilham, R. B. Luftig, M. J. McDowell, T. Matsuhisa, J. L. Nichols, D. M. Pett, J. J. Skehel, R. E. Smith, T. C. Vanaman and H. J. Zweerink. This work was supported by grants no. AI-08909 and 5 SO4 RR 06148 from the United States Public Health Service, grant no. AT-(40-1)-3857 from the United States Atomic Energy Commission, and grant no. GB-8077 from the National Science Foundation.

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SYNTHESIS BY REVERSE TRANSCRIPTASE OF DNA COMPLEMENTARY TO GLOBIN MESSENGER RNA

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CONTENTS

Synthesis by reverse transcriptase of DNA complementary to globin messenger RNA

I. Introduction

RNA tumor viruses contain a DNA polymerase which will synthesize a faithful DNA copy of viral RNA (BALTIMORE, 1970; TEMIN and MIZUTANI, 1970; SPIEGELMAN *et al.*, 1970 a,b; ROKUTANDA *et al.*, 1970; DUESBERG and CANAANI, 1970; TEMIN and BALTIMORE, 1972). This enzyme is easily released and purified from virions and will utilize a wide variety of polymers as templates (VERMA *et al.*, 1971; KACIAN *et al.*, 1971; DUESBERG *et al.*, 1971 a,b; HURWITZ and LEIS, 1972; LEIS AND HURWITZ, 1972; VERMA and BALTIMORE, 1973). In order for a template to be copied; a primer or initiator is required which binds to the template by hydrogen bonds (BALTIMORE and SMOLER, 1971). The 3'-OH end of the primer is then covalently attached to the newly synthesized DNA (SMOLER *et al.*, 1971). When the 60-70S tumor viral RNA is transcribed, the primer is apparently a short polyribonucleotide which is found attached to the DNA product (VERMA *et al.*, 1971; LEIS and HURWITZ, 1972; FLÜGEL and WELLS, 1972; VERMA *et al.*, 1972b).

One use of the RNA tumor virus DNA polymerase could be the synthesis of DNA complementary to messenger RNA (SPIEGELMAN *et al.*, 1971). Most eukaryotic messenger RNAs (mRNA) contain adenine-rich sequences [poly(A)] as an integral part of their structure [LIM and CANELLAKIS, 1970; KATES, 1970; PHILIPSON *et al.*, 1971; FIRTEL *et al.*, 1972; for an up-to-date reference list of poly(A) containing metazoan genetic messages see SLATER *et al.*, 1972]. Because these sequences are apparently present at or near the 3'-end of the mRNA, an oligomer of dT which can hydrogen bond to the poly(A) segment can be used as a primer for transcription of mRNA into DNA. The mRNA for rabbit globin can be partially purified from rabbit reticulocytes (LABRIE, 1969; LOCKARD and LINGREL, 1969; HOUSMAN *et al.*, 1971) by sucrose gradient rate-zonal centrifugation. The identity of the RNA recovered from the

10S region has been established by its ability to direct synthesis of globin in a cell-free protein-synthesizing system (LOCKARD and LINGREL, 1969; HOUSMAN *et al.*, 1971). It contains poly(A) sequences, at least some of which are at the 3'-end of the molecule (LIM and CANELLAKIS, 1970; BURR and LINGREL, 1971). Reticulocyte 10S RNA would therefore appear to be a good model RNA for transcription into DNA by the tumor virus DNA polymerase. The successful synthesis of DNA complementary to 10S RNA can be achieved (VERMA *et al.*, 1972; KACIAN *et al.*, 1972; and ROSS *et al.*, 1972), as described diagrammatically in fig. 1. Table I describes the requirements for DNA synthesis from 10S RNA. Following are some of the salient features of this system:

a) The synthesis of complementary DNA is primer-dependent.
b) The DNA synthesized is approximately 450 nucleotides long.
c) The DNA transcript is a faithful copy of 10S RNA, because it will hybridize to the 10S RNA.
d) If actinomycin D is omitted from the reaction mixture, double-stranded DNA is obtained, as characterized by its resistance to single-strand specific nuclease (FAN and BALTIMORE, 1973).
e) Both the single-stranded and double-stranded DNA products can be transcribed back into RNA by *Escherichia coli* RNA polymerase.
f) Isolated single-stranded DNA can be used as a template for further DNA synthesis. The newly synthesized DNA is covalently linked to the template. The product is mostly double-stranded.

In this symposium we shall describe some of these properties in detail.

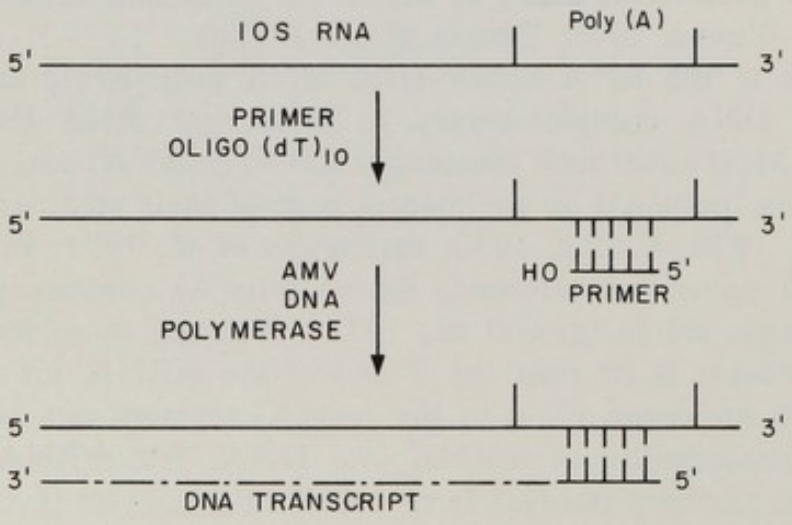

FIG. 1. Diagrammatic model for the synthesis of complementary DNA from 10S globin messenger RNA.

Table I. Requirements for DNA synthesis using 10S reticulocyte RNA as template.

Exp. no.	Reaction mixture	pmol dGMP incorporated in 90 min
1	Complete mixture	225
	Without $(dT)_{10}$	10
	With ribonuclease	2
	With actinomycin D	150
2	Complete mixture	90
	Without dTTP	3
	Without dCTP	3
	Without dATP	4
3	Complete mixture	112
	Without $(dT)_{10}$, with $(dT)_{12-16}$	119
	Without $(dT)_{10}$, with $(dG)_{12-16}$	39
	Without $(dT)_{10}$, with $(dC)_{12-16}$	8
	Without $(dT)_{10}$, with $(dA)_{12-16}$	9

The complete reaction mixture consisted of the following in 0.1 ml: 50 mM Tris HCl (pH 8.3), 10 mM dithiothreitol, 6 mM magnesium acetate, 60 mM NaCl, 20 μg/ml actinomycin D, 1 mM dATP, 1 mM dCTP, 1 mM dTTP, 160 μM ^{3}H-dGTP (40 cpm/pmol), 14.2 pmol $(dT)_{10}$ (concentrations given in terms of monomer concentration), 1,000 pmol rabbit reticulocyte 10S RNA, and 0.20-0.50 μg of AMV DNA polymerase (VERMA and BALTIMORE, 1973). For experiment 1, actinomycin was omitted from the reaction mixture except where indicated. Ribonuclease-treated samples were prepared by diluting 2 μl of sample containing 1,000 pmol of rabbit reticulocyte 10S RNA to 10 μl with 0.01 M Tris-HCl, pH 7.6, and 0.01 M NaCl and adding 2 μl of ribonuclease reagent. The ribonuclease reagent contained 400 μg of pancreatic ribonuclease A/ml (Worthington Biochemical), 80 μg of ribonuclease T_1/ml (Calbiochem, 5,000 U/mg) and 1 mg of bovine serum albumin/ml in 0.01 M Tris-HCl (pH 7.6) and 0.01 M NaCl. The samples were incubated at 37 °C for 30 min and the RNA was then used in a standard reaction mixture. In experiment 3, we compared $(dT)_{10}$ and $(dT)_{12-16}$, because they came from different sources. The amounts of $(dT)_{12-16}$, $(dG)_{12-16}$, $(dC)_{12-16}$ and $(dA)_{12-16}$ used in experiment 3 were 1,420 pmol of nucleotides/reaction mixture. Reactions were carried out in sealed tubes under an N_2 atmosphere, and incubated at 37 °C for 90 min. Acid-precipitable radioactivity was determined as previously described (BALTIMORE *et al.*, 1970).

II. Requirement of Primer

It is clear from table I that in the absence of primer, there is little detectable synthesis of complementary DNA. Although oligo(dT) is the most efficient primer, it can be replaced by a less efficient primer, oligo(dG). Figure 2a compares the rate of synthesis of DNA using oligo(dT) or oligo(dG) primers. The oligo(dT)-stimulated reaction reaches saturation in 90-120 minutes, whereas the oligo(dG)-stimulated reaction proceeds at a slower rate. At saturation, about 40% of input nucleotides of the template are transcribed into complementary DNA with oligo(dG) primer. Under similar conditions, using oligo(dT), about 60% of the input nucleotides are transcribed into DNA. The respective sizes of oligo(dT)- and oligo(dG)-stimulated complementary DNA are compared in fig. 2b. Oligo(dG)-primed DNA is slightly smaller in size and more heteroge-

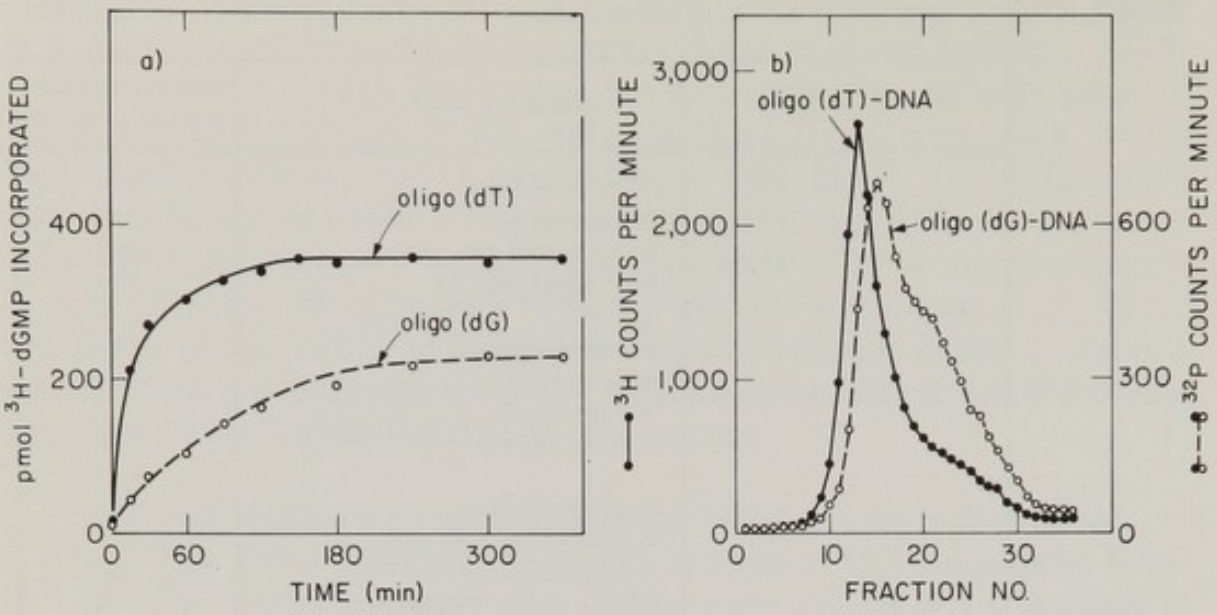

FIG. 2a. Kinetics of synthesis of DNA using oligomers of dT and dG as primers. The complete reaction mixture described in table I was used with 1600 pmol of rabbit reticulocyte RNA. The amount of oligo(dT) and oligo(dG) primer used was 100 pmol (concentration given in terms of monomer concentration). In all experiments, commercial oligo(dT)$_{15}$ and oligo(dG)$_{15}$ were used as primers. Actinomycin D was present at a concentration of 100 μg/ml. Reactions were carried out for various times as indicated. Closed circles= oligo(dT)-stimulated DNA; open circles= oligo(dG)-stimulated DNA.

FIG. 2b. Size comparison of oligo(dT)- and oligo(dG)-stimulated DNA on alkaline sucrose gradients. Linear sucrose gradients (5-20%) containing 0.7 M NaCl, 0.3 M NaOH, 0.005 M EDTA, pH 12.6, were prepared. ^{3}H-labeled oligo(dT)-DNA and ^{32}P-labeled oligo(dG)-DNA were made 0.3 M with NaOH and boiled for 5 minutes at 100 °C. The product was neutralized with 3 N HCl and purified from unincorporated radioactivity by gel filtration on G-50 Sephadex columns as described earlier (VERMA *et al.*, 1971). ^{3}H-labeled oligo(dT)-DNA and ^{32}P-labeled oligo(dG)-DNA were made up to 0.1 ml in gradient buffer and layered on the gradient. The gradients were centrifuged at 45,000 rpm in an SW 50.1 rotor for 16 hours at 4 °C. Two drop fractions were collected by puncturing the bottom of the gradient tube and were neutralized by adding 1 N acetic acid followed by 3.0 ml of distilled water and 10.0 ml of Aquasol (from New England Nuclear) and were shaken thoroughly to make a gel. Closed circles= ^{3}H-oligo(dT)-DNA; open circles= ^{32}P-oligo(dG)-DNA.

nous. Oligo(dG)-DNA, upon hybridization to 10S globin RNA, is rendered completely resistant to the S_1 nuclease from *Aspergillus*, which degrades single-stranded DNA selectively. In order to eliminate the possibility that oligo(dG) serves as primer for the transcription of either α- or β-10S RNA exclusively, we compared the stimulation of DNA synthesis with oligo(dG) and oligo(dT) using isolated α- and β-10S RNA from rabbit reticulocytes (TEMPLE and HOUSMAN, 1972). Table II shows that both α- and β-10S RNA are transcribed with the same efficiency using oligo(dG) and oligo(dT) primers; β-10S RNA appears to be transcribed more extensively than α-10S RNA. Table II also shows that oligo(dG) does not stimulate any DNA synthesis when 28S and 18S RNA

Table II.

Template	Primer	pmol dGMP incorporation	% of input nucleotides transcribed
Rabbit reticulocyte 10S RNA	oligo(dT)	350	58
	oligo(dG)	235	39
Rabbit reticulocyte α-10S RNA	oligo(dT)	80	13
	oligo(dG)	68	11
Rabbit reticulocyte β-10S RNA	oligo(dT)	216	36
	oligo(dG)	192	32
Rabbit reticulocyte 28S RNA		6	< 2
	oligo(dG)	8	< 2
	oligo(dT)	9	< 2
Rabbit reticulocyte 18S RNA		4	< 2
	oligo(dG)	2	< 1
	oligo(dT)	7	< 2

The complete reaction mixture as described in table I and legend to fig. 2a, was used. The concentration of rabbit reticulocyte 10S RNA was 2400 pmol; α-10S RNA, 2400 pmol; β-10S RNA, 2400 pmol; rabbit reticulocyte 28S RNA, 1700 pmol; and rabbit reticulocyte 18S RNA, was 1500 pmol. The % input nucleotides transcribed has been determined by multiplying pmol of dGMP incorporated times four (assuming that all 4 deoxyribonucleoside triphosphates have been incorporated in equimolar ratios).

from rabbit reticulocytes are presented as templates. Thus, like oligo(dT) (table I), oligo(dG) specifically utilized the 10S RNA from rabbit reticulocytes as template to synthesize complementary DNA.

The role of oligo(dG) as primer for the synthesis of complementary DNA is puzzling because there are no known poly(C)-rich regions in the 10S RNA. However, regions of RNA containing 5 to 6 residues of cytidylic acid could well exist and act as sites for primer attachment. Because the oligo(dG)-DNA is quite long, some of the primer binding sites must be near the 3'-end of the template. Thus, it might be expected that oligo(dT)-primed DNA synthesis, which starts at the 3'-end, would cover the oligo(dG) binding sites and prevent any oligo(dG)-stimulated synthesis. This conjecture has been confirmed by showing that addition of oligo(dG) at the end of an oligo(dT)-primed synthesis leads to little further DNA synthesis. If, however, oligo(dT) is added after maximal oligo(dG)-stimulated synthesis has occurred, further stimulation of DNA synthesis is detected.

If oligo(dT)-DNA is a nearly complete transcript of 10S RNA, then the sum total of the sizes of oligo(dG)-DNA and residual oligo(dT)-DNA [i.e., where oligo(dG) is used as primer and at the end of the incubation, oligo(dT) is added] should not exceed the size of oligo(dT)-DNA. Figure 3 portrays sedimentation patterns of oligo(dG)-DNA and residual oligo(dT)-DNA on alkaline sucrose gradients. By using the method of STUDIER (1965), the size of the largest species of residual oligo(dT)-DNA was determined to be approximately 150 to 160 nucleotides long and

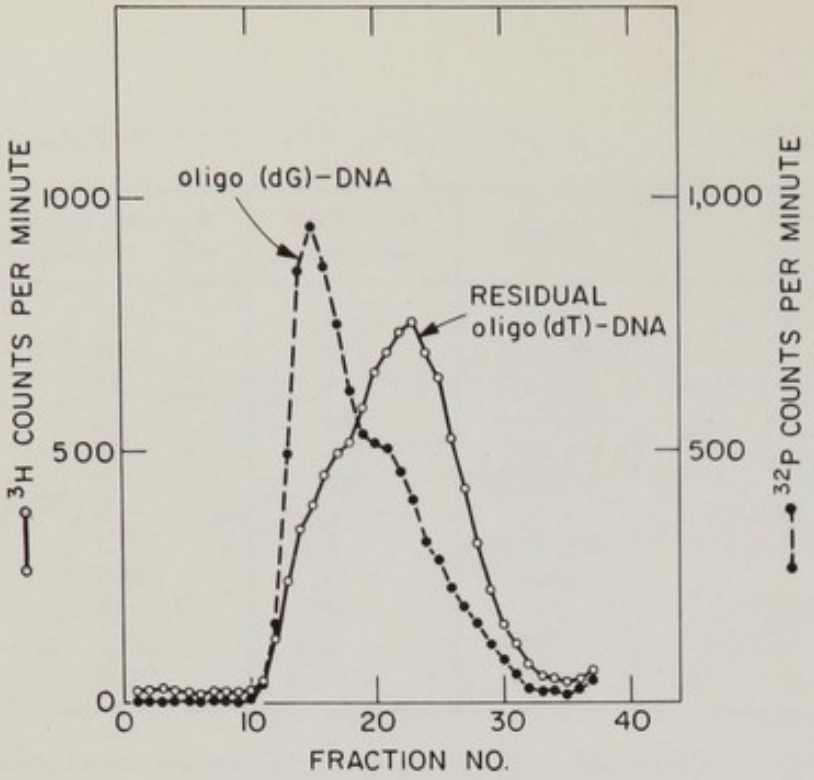

FIG. 3. Size comparison of oligo(dG)-DNA and residual oligo(dT)-DNA on alkaline sucrose gradients. Oligo(dG)-primed reaction mixture (as in fig. 2a) was incubated for seven hours and then oligo(dT) (as in fig. 2a) was added along with ^{3}H-labeled deoxyguanosine triphosphate (600 cpm/pmol). The reaction was further incubated for 180 minutes, and the product purified by gel filtration as described before. The product was analyzed by centrifugation on alkaline sucrose gradient as described in fig. 2b along with ^{32}P-labeled oligo(dG)-DNA as marker. Open circles= ^{3}H-residual oligo(dT)-DNA; closed circles= ^{32}P-oligo(dG)-DNA.

that of oligo(dG)-DNA to be approximately 350 to 370 nucleotides long. Together they represent a size approximately 500 to 550 nucleotides long as compared to oligo(dT)-DNA which is approximately 450 nucleotides long. Thus the size of oligo(dG)-DNA plus residual oligo(dT)-DNA is approximately 10 to 20% larger than oligo(dT)-DNA alone. This suggests that some nucleotide sequences in oligo(dG)-DNA are unique. In order to investigate this futher, competition hybridization studies were carried out, and the results show that approximately 30% of the sequences in oligo(dG)-DNA do not compete with oligo(dT)-DNA and appear to be unique. So, it appears that oligo(dT)-primed DNA synthesis is initiated near the 3'-end of the RNA while oligo(dG)-stimulated DNA synthesis is initiated from regions [presumably oligo(C)-rich] situated internally in the RNA, and the DNA transcript includes some sequences analogous to oligo(dT)-DNA and some unique sequences. A tentative model to explain oligo(dT)- and oligo(dG)-primed synthesis of comple-

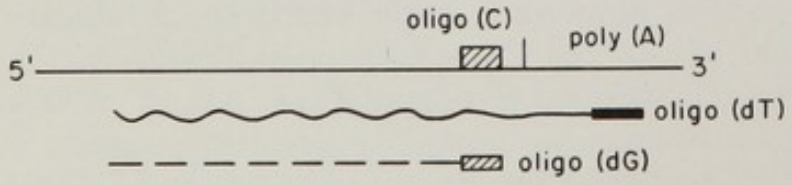

 FIG. 4. A tentative model to explain the synthesis of DNA by oligo(dG) primer.

mentary DNA is proposed in fig. 4. This model can explain satisfactorily a number of the properties of the oligo(dG)-stimulated reaction: a) The slower kinetics of oligo(dG)-primed DNA would be a result of the binding of oligo(dG) to short cytidylic acid-rich regions which would not be very stable; b) that at saturation, oligo(dG)-DNA is only slightly shorter than oligo(dT)-DNA; c) if oligo(dG) is added to oligo(dT)-primed reaction mixture, no detectable enhancement of incorporation is observed. Conversely, if oligo(dT) is added to a reaction mixture which has previously been incubated with oligo(dG), significant enhancement in incorporation is observed; d) the sum total of the sizes of oligo(dG)-DNA and residual oligo(dT)-DNA barely exceeds the size of oligo(dT)-DNA. The model is, however, unable to explain competition hybridization results which suggest that either the oligo(dG)-DNA is transcribed more extensively near the 5'-end of the 10S RNA or that oligo(dG) is priming the transcription of some unknown species of RNA present in the 10S RNA preparation.

III. Transcription of RNA from Complementary Globin DNA

The DNA transcript of 10S RNA can be used for RNA synthesis *in vitro* by *E. coli* RNA polymerase. Fig. 5 shows the rate of incorporation of GMP into acid-insoluble material. Table III summarizes the require-

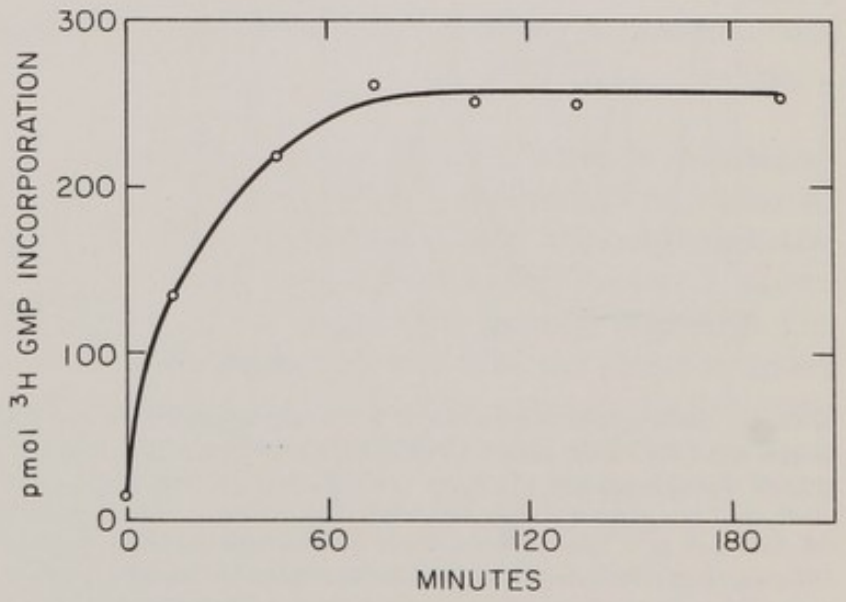

FIG. 5. Kinetics of synthesis of RNA from single-stranded globin DNA template. The complete reaction mixture consisted of the following in 0.1 ml: 50 mM Tris-HCl (pH 7.9), 50 mM magnesium acetate, 0.4 mM dithiothreitol, 150 mM KCl, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 160 μM ^{3}HGTP (40 cpm/pmol), 1500 pmol of single-stranded globin DNA and 10 μg of *E. coli* RNA polymerase (gift of Dr. R. A. FIRTEL). The reaction mixture was incubated at 37 °C and aliquots of 10 μl were withdrawn at the times indicated. Acid-precipitable radioactivity was determined as described before (table I).

Table III. Requirements for RNA synthesis using 8S single-stranded globin DNA.

Reaction mixture	pmol of GMP incorporated
Experiment I	
Complete	32
Without ATP	6
Without CTP	4
Without UTP	3
Experiment II	
Complete	310
Rifampicin (20 μg/ml)	50

The complete reaction mixture as described in fig. 5 was used, except 200 pmoles of 8S single-stranded globin DNA in Expt. I and 1600 pmoles in Expt. II were added. Reactions were carried out in sealed tubes under an N_2 atmosphere, and incubated at 37 °C for 120 minutes. Acid-precipitable radioactivity was determined as previously described.

ments for RNA synthesis. The reaction requires all 4 ribonucleoside triphosphates for synthesis of RNA. Rifampicin (20 μg/ml) inhibits about 85% of RNA synthesis. Both oligo(dT)-DNA and oligo(dG)-DNA can act as templates for the synthesis of RNA. Figs. 6a and b depict the

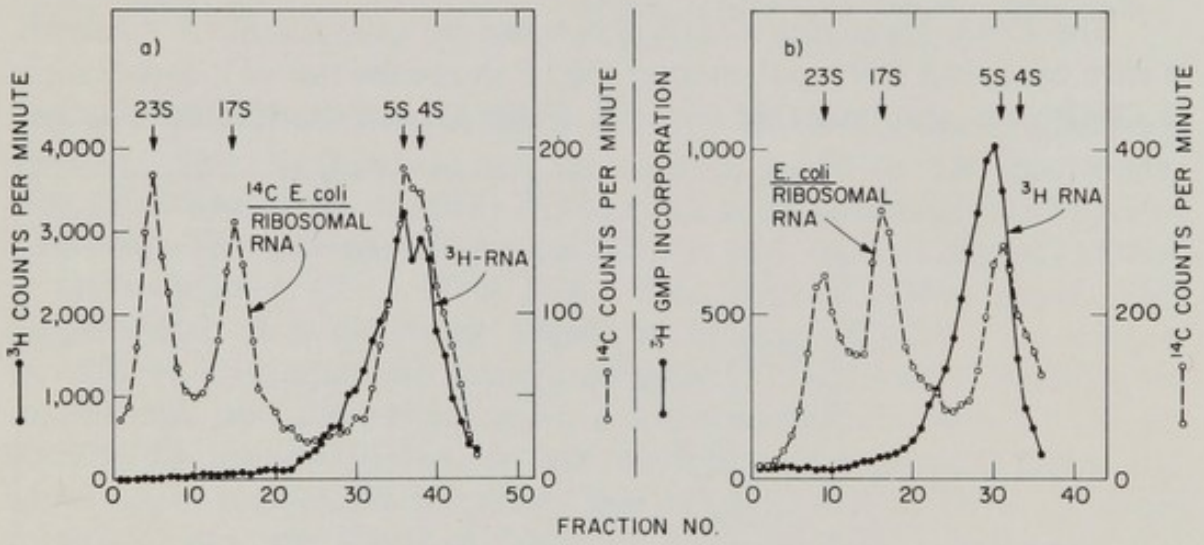

FIG. 6. Sedimentation patterns of RNA synthesized from oligo(dT)-and oligo(dG)-stimulated single-stranded rabbit globin DNA. The complete reaction mixture described in fig. 5 was used except that the specific activity of ^{3}HGTP was 600 cpm/pmol, and the amount of DNA used in each case was approximately 800 pmol. The reactions were terminated at 180 minutes by addition of Sarkosyl at a final concentration of 1%. The products were purified over Sephadex G-75 columns from the unincorporated radioactivity. The column-purified products were then treated with deoxyribonuclease (Worthington) at a concentration of 10 μg/ml in a buffer containing 1 mM Mg^{2+} and incubated at 37° C for 10 minutes. The DNase was inactivated by adding 10 mM EDTA and diluted in the gradient buffer. Two 4.8 ml, 5-20% sucrose gradients made in 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5) and 0.001 M EDTA were prepared. The products in a volume of 100 μl were layered separately on two gradients along with ^{14}C-labeled *E. coli* ribosomal RNA as marker (provided by Dr. A. JACOBSON). The gradients were centrifuged for 4-1/2 hours at 45,000 rpm at 4 °C in an SW 50.1 rotor. Gradients were collected and radioactivity counted as described in fig. 2b. Closed circles= ^{3}H-RNA; open circles= ^{14}C-*E. coli* ribosomal RNA.

sedimentation patterns on neutral sucrose gradients of the RNA synthesized using oligo(dT)-DNA and oligo(dG)-DNA templates, respectively. Although some of the RNA synthesized sediments faster, the majority of the RNA synthesized sediments between 5 and 6S.

Using the complementary DNA made from 10S RNA isolated from patients with sickle-cell anemia, and separate reaction mixtures containing one of the four α-P^{32} labeled nucleoside triphosphates, ^{32}P-labeled RNA sedimenting at 6S in DMSO gradients has been obtained (FORGET *et al.*, 1972; MAROTTA *et al.*, 1973). The T_1 ribonuclease fingerprint pattern of the purified ^{32}P-labeled synthetic 6S RNA is compatible with the fingerprint of original 10S RNA which has been treated with T_1-RNase and then with polynucleotide kinase. Many T_1-RNase sequences were identical with oligopeptides found in the α and β chains of globin. Similar comparisons are in progress with RNA synthesized from DNA complementary to rabbit reticulocyte 10S RNA.

The majority of the RNA synthesized from oligo(dT)-stimulated single-stranded rabbit globin DNA hybridizes back to the template DNA and is efficiently competed out with excess cold 10S RNA. Double-stranded DNA made in the absence of actinomycin D is also a good template for RNA synthesis, except that the size of the RNA synthesized is much smaller. Detailed studies of the hybridization properties of these synthetic RNAs are in progress.

IV. Synthesis of Double-Stranded DNA from the Single-Stranded Globin DNA Transcript

If actinomycin D is omitted from the reaction mixture containing globin mRNA (table I), double-stranded DNA is synthesized as measured by enhanced incorporation of deoxyribonucleoside triphosphates and resistance to single-stranded DNA-specific nuclease. Figure 7 shows the analysis of double-stranded DNA on alkaline sucrose gradients. The double-stranded DNA sediments slightly slower than the single-stranded DNA made in the presence of actinomycin D. This suggests the following 3 mechanisms of synthesis of double-stranded DNA: a) There is some primer attached at or near the 3'-OH end of the single-stranded DNA which serves as initiator for the synthesis of the second strand of DNA; b) the 3'-OH end of nascent single-stranded DNA chains supports the synthesis of the second strand; and c) the synthesis of the second strand of DNA does not require a primer.

In order to rule out the possibility that some primer is fortuitously attached at the 3'-OH end of the single-stranded DNA, we purified the single-stranded DNA made in the presence of actinomycin D on alkaline sucrose gradients. Peak fractions were pooled and excess sucrose and

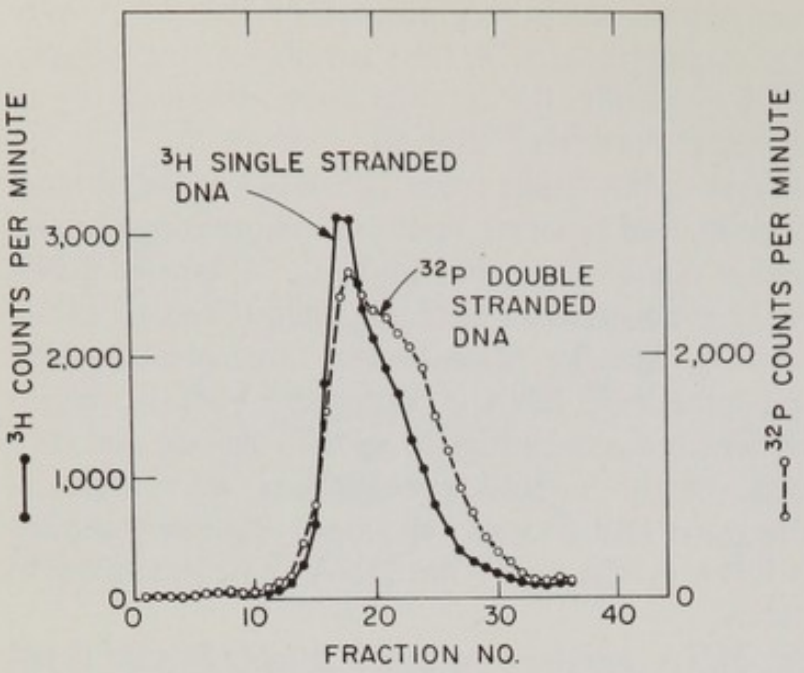

FIG. 7. Alkaline sucrose gradient profile of double-stranded DNA made from 10S rabbit reticulocyte RNA in the absence of actinomycin D. The complete reaction mixture as described in table I was used except that actinomycin D was omitted and ^{3}HdGTP was replaced with ^{32}PdGTP. The reaction was carried out for 6 hours, and the product was purified by gel filtration and analyzed on 5-20% alkaline sucrose gradient as described in fig. 2b. Closed circles= ^{3}H-labeled single-stranded globin DNA marker; open circles= ^{32}P-labeled double-stranded DNA.

alkali removed by gel filtrations on G-50 Sephadex columns. This is referred to as purified single-stranded DNA. When purified single-stranded DNA is incubated with 4 deoxyribonucleoside triphosphates and purified avian myeloblastosis virus (AMV) DNA polymerase, double-stranded DNA is synthesized. The product has been analyzed on alkaline sucrose gradients. Figs. 8a and b show the profile of ^{3}H-labeled single-stranded DNA before incubation and after incubation with ^{32}P-labeled deoxyribonucleoside triphosphates. The ^{32}P-labeled second strand of DNA sedimented faster than the majority of ^{3}H-labeled single-stranded DNA template. About 20-30% of the input nucleotides of the template strand were transcribed, and about that amount of ^{3}H-labeled DNA sedimented coincidentally with ^{32}P-labeled DNA. Since the new strand of DNA sedimented faster than the template strand and carried with it some template strand, it appears that the second strand was covalently linked to the 3'-OH end of the template strand.

When the product was chromatographed on hydroxyapatite columns, the majority of the unreacted template strand was eluated by 0.12 M phosphate buffer, whereas the ^{3}H— and ^{32}P-covalently linked double-stranded DNA eluated at 0.48 M phosphate buffer concentration. S_1 nuclease digestion of the 0.48 M phosphate buffer eluate of hydroxyapatite columns showed that 100% of ^{32}P-label remained acid-insoluble, whereas only 70% of the ^{3}H-label remained acid-precipitable. This suggests

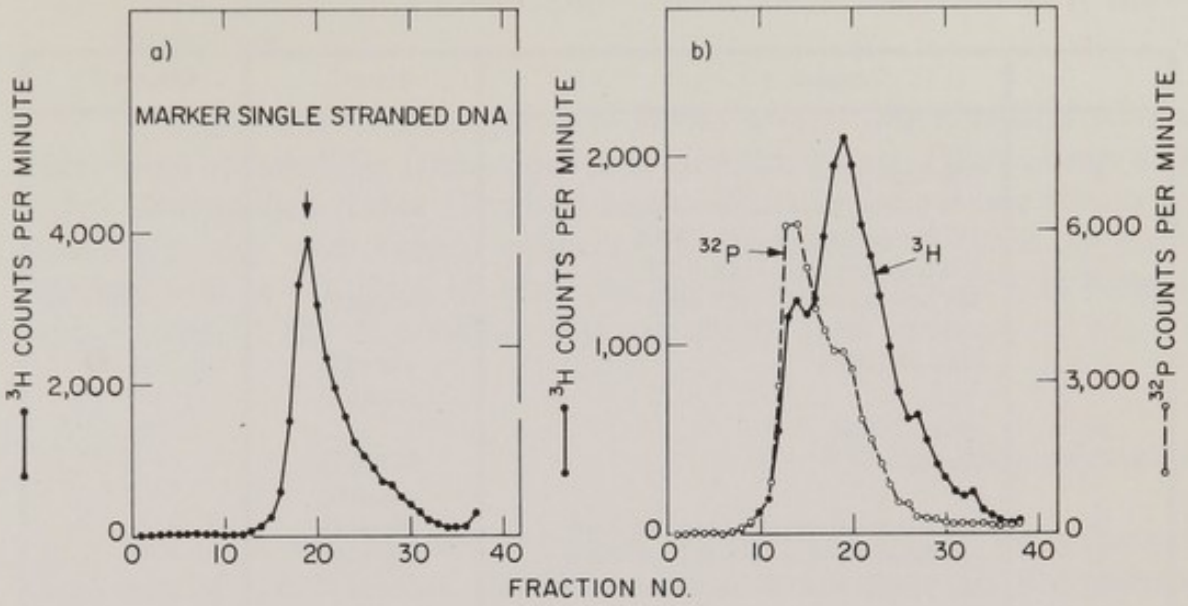

FIG. 8. Sedimentation profiles of purified single-stranded globin DNA before and after incubation with ^{32}P-labeled deoxyribonucleoside triphosphates. Single-stranded DNA was made as described in table I and then purified further by sedimentation on an alkaline sucrose gradients. Peak fractions were collected and excess alkali and sucrose removed by gel filtration. Panel (a) shows the sedimentation profile. The arrow shows the position of a marker ^{32}P-labeled 8S single-stranded DNA. Panel (b) depicts the sedimentation pattern after incubating 2100 pmoles of ^{3}H-labeled purified single-stranded DNA (1236 cpm/pmol of dGMP) with 50 mM Tris-HCl (pH 8.3), 5 mM dithiothreitol, 6 mM magnesium acetate, 60 mM NaCl, 0.6 mM each of dATP, dCTP and dTTP, ^{32}P-labeled dGTP (spec. act. 12,000 cpm/pmol), and AMV DNA polymerase for 6 hours at 37 °C. The product was purified by gel filtration and part of it analyzed on alkaline sucrose gradients described in fig. 2b. Closed circles= ^{3}H single-strand globin DNA; open circles= ^{32}P-labeled second strand of DNA.

that the ^{3}H-labeled template strand was not completely copied.

Analysis of the product by formamide gel electrophoresis showed that the ^{3}H- and ^{32}P-labeled double-stranded DNA migrated more slowly than the marker ^{32}P-labeled single-stranded DNA (figure not shown), suggesting that the double-stranded DNA was larger in size than the single-stranded DNA.

Thus, from the above results, it appears that: a) the purified single-stranded DNA acts as a template for the synthesis of a second strand of DNA; b) the second strand is covalently linked to the template strand; and c) the second strand is not a complete transcript of the complete strand.

As mentioned before, double-stranded DNA synthesized in the absence of actinomycin D is smaller in size than the single-stranded DNA. We are now investigating this reaction in detail to determine if the nascent growing chain of DNA forms a "hair pin" and provides the 3'-OH end for the synthesis of the second strand or whether there is another mechanism of synthesis of this double-stranded DNA.

Table IV.

	Template	Primer[1]	Efficiency[2]
1.	70S AMV RNA		+ +
2.	70S hamster leukemia virus RNA		+ +
3.	70S murine leukemia virus RNA		+ +
4.	35S heat-denatured AMV RNA		+
	35S heat-denatured AMV RNA	oligo(dT)	+ +
5.	polio 35S RNA		+
	polio 35S RNA	oligo(dT)	+ +
		oligo(dG)	+ +
6.	phage f2 RNA		+
	phage f2 RNA	oligo(dT)	+ +
		oligo(dG)	+ +
7.	28S rabbit reticulocyte ribosomal RNA	oligo(dT)	+
8.	18S rabbit reticulocyte ribosomal RNA	oligo(dT)	+
9.	10S rabbit, duck or human reticulocyte RNA		+
	10S rabbit, duck or human reticulocyte RNA	oligo(dT)	+ + +
	10S rabbit, duck or human reticulocyte RNA	oligo(dG)	+ + +
10.	26S myosin RNA		+
	26S myosin RNA	oligo(dT)	+ +
11.	10S histone RNA		+
	10S histone RNA	oligo(dT)	+ +
12.	silkworm mRNA		+
	silkworm mRNA	oligo(dT)	+ +
		oligo(dC)	+ +
13.	14S crystallin lens mRNA		+
	14S crystallin lens mRNA	oligo(dT)	+ + +
14.	*E. coli* 5S RNA		+
	E. coli 5S RNA	oligo(dT)	+
		oligo(dG)	+
15.	yeast-fmet-tRNA	oligo(dT)	+
16.	crude *E. coli* 4S RNA	oligo(dT)	+
17.	slime mold mRNA	oligo(dT)	+ + +
18.[3]	vaccinia mRNA	oligo(dT)	+ + +
19.	single-stranded 8S globin DNA		+ + +
20.	commercial calf thymus and salmon sperm DNA		+ + +

[1] The oligomer primers have a chain length of 12 to 18 nucleotides.

[2] + + + = Excellent template, approximately 20-80% of nucleotides of the input template are transcribed. + + = Fair template, approximately 1-20% of nucleotides of the input template are transcribed. + = Poor template, less than 1% of nucleotides of the input template are transcribed.

[3] ZASSENHAUS and KATES (1972).

The conditions employed are the same as described for the assay of the fractions, except for substituting appropriate template and primer. The incubation time is 90 to 120 minutes. This represents the yield but not the rate of reaction. The change of ionic milieu can alter the efficiency of certain templates.

V. Transcription of Various Other RNA-Templates by AMV DNA Polymerase

In addition to reticulocyte 10S RNA, several other natural RNAs have been successfully transcribed into complementary DNA in our and other laboratories. Table IV tabulates the efficiency of various RNAs as templates with and without primers. The properties of some of these systems will be described in detail elsewhere (BERNS *et al.*, 1973; VERMA and FIRTEL, 1973, manuscripts in preparation).

VI. Acknowledgments

This work was supported by a contract from the Special Virus Cancer Program of the National Cancer Institute awarded to D. BALTIMORE and by N.I.H. grant no. GM-17151 awarded to Dr. U. L. RAJBHANDARY. I.M.V. and G.F.T. were fellows of the Jane Coffin Childs Memorial Fund for Medical Research. H.F. was a Helen Hay Whitney fellow and D.B. was an American Cancer Society Professor of Microbiology.

The avian myeloblastosis virus was generously supplied by Dr. J. BEARD.

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TEMPERATURE-SENSITIVE MUTANTS OF HERPES SIMPLEX VIRUS[1]

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CONTENTS

Temperature-sensitive mutants of herpes simplex virus[1]

I. Introduction

The advances made in recent years in the field of animal virus genetics stem from two main developments: (1) the elegant use of conditional-lethal mutants of bacteriophage T4 for genetic mapping and for gene functional analysis (EPSTEIN *et al.*, 1963), and (2) the development of a plaque assay for precise quantification and cloning of animal viruses (DULBECCO, 1952). In bacteriophage genetics, conditional-lethal mutants of the suppressor type (amber mutants) have been of greater advantage than temperature-sensitive (ts) mutants. On the other hand, genetic work with animal viruses has featured the use of ts mutants, mainly because of the difficulty in recognizing suppressor sensitive animal cells necessary for the isolation of suppressor type mutants.

Temperature-sensitive mutants of a number of different animal viruses have been used to advantage in viral genome analysis and the definition of viral gene functions (COOPER, 1967; FENNER, 1969, 1970). Unlike wild-type (WT) viruses which replicate equally well at low ("permissive") and high ("nonpermissive") temperatures, ts mutants, derived from them by different mutagenic agents, are capable of replication only at low temperatures. These mutants result from mis-sense mutations which can probably occur in all viral genes. However, only those mutants with changes in functions essential for virus replication will be recognized by the selective procedures used. At a molecular level, these mutants contain an altered amino acid sequence in an essential virus-coded protein which renders the protein unable to assume and/or maintain its functional

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configuration at the restrictive temperature and thus they are defective for growth (FENNER, 1969). Theoretically, ts mutations should affect most of the essential structural and nonstructural viral proteins. Thus the availability of ts mutants of animal viruses offers a possibility not only for the construction of genetic maps but also for the study of the role that specific viral gene products play in virus replication.

Herpes simplex virus (HSV) is a member of a widespread group of relatively large DNA viruses, some of which have been recently associated with malignancies in man and animals (KLEIN, 1972). Two subtypes of HSV are recognized, type 1 (HSV-1) and type 2 (HSV-2), which are genetically related (BRONSON *et al.*, 1972; KIEFF *et al.*, 1972; LUDWIG *et al.*, 1972) but differ in certain biologic and immunologic properties (EJERCITO *et al.*, 1968; NAHMIAS *et al.*, 1968; FIGUEROA and RAWLS, 1969; PLUMMER *et al.*, 1970). Virologic evidence has indicated a close association between HSV-2 and cervical cancer (RAWLS *et al.*, 1969; NAHMIAS *et al.*, 1970; ROYSTON and AURELIAN, 1970; FRENKEL *et al.*, 1972). Malignant transformation of hamster cells has been attained with HSV-2 (DUFF and RAPP, 1971); there is no substantial data to implicate HSV-1 in oncogenesis; however, it appears that the virus under special conditions can transform hamster cells in tissue culture (RAPP and DUFF, 1973).

The DNA of HSV has a molecular weight of about 100×10^6 daltons (BECKER *et al.*, 1968; KIEFF *et al.*, 1971; GRAHAM *et al.*, 1972) and thus has sufficient information to code for about 50,000 amino acids. As many as 24 HSV-1 structural polypeptides have been identified to date by polyacrylamide gel electrophoresis, ranging in molecular weight from 25,000 to 257,000 daltons (SPEAR and ROIZMAN, 1972). From a consideration of the finding that HSV-1 DNA is free of repetitive sequences (FRENKEL and ROIZMAN, 1971) and assuming that (1) viral messenger RNA (mRNA) is transcribed asymmetrically and is not complementary, and (2) the larger proteins do not represent aggregates of two or more polypeptides; it has been estimated that the sum of the molecular weights of the 24 structural polypeptides of 2.58×10^6 daltons (25,800 amino acids) represents about 50% of the coding capacity of the HSV-1 genome (SPEAR and ROIZMAN, 1972). Even though these estimates are tentative, such a high proportion of HSV-1 genes coding for structural polypeptides should insure the isolation of ts mutants defective in structural components. On the other band, if these estimates are correct, about 50% of the HSV-1 genome may code for nonstructural proteins directly or indirectly functional in virus replication. A major feature in infection of cells by HSV is the early synthesis of enzymes associated with DNA synthesis (KEIR, 1968; KIT and DUBBS, 1969; HAY *et al.*, 1971). Biochemical and immunologic evidence indicates that of these, thymidine kinase and DNA polymerase are different from the cellular enzymes and thus are coded

for by the viral genome (KEIR and GOLD, 1963; KIT *et al.*, 1967; KLEMPERER *et al.*, 1967; KEIR, 1968; KIT and DUBBS, 1969). It remains to be seen whether DNA exonuclease (KEIR and GOLD, 1963; MORRISON and KEIR, 1968) and the more recently described ribonucleotide reductase (COHEN, 1972) activities in HSV-infected cells are virus-coded. The same holds true for a protein kinase activity associated with enveloped herpes simplex virions (RUBENSTEIN *et al.*, 1972). Isolation and characterization of sufficient numbers of ts mutants of HSV should lead to an answer to this question and hopefully to the identification of the genes coding for those enzymes that are indeed of viral origin.

Several other aspects of the composition and replication of HSV DNA are of importance in considering genetic work with ts virus mutants. Evidence that the genome of both HSV-1 and HSV-2 contains single-stranded nicks has been obtained by fragmentation of denatured virion DNA into 6 fragments of non-random size and unique nucleotide sequence upon centrifugation in alkaline sucrose density gradients (FRENKEL and ROIZMAN, 1972). Furthermore, it appears that newly synthesized HSV DNA extracted from nuclei of infected cells yields a much larger number of fragments than that extracted from the mature virion and that with time after infection fragment elongation occurs involving repair and/or ligation (FRENKEL and ROIZMAN, 1972). These findings are of importance in terms of the problem of marker reassortment in recombination experiments with ts mutants of HSV. Another aspect of ts mutant work with HSV lies in the identification of genes specific for HSV-1 and HSV-2 (in particular the genes responsible for the potential oncogenicity of HSV-2) and those shared by the two virus types. The DNA of the two strains cannot be differentiated on the basis of molecular weight (KIEFF *et al.*, 1971; GRAHAM *et al.*, 1972). However, a difference in base composition of 2 mole percent G+C has been consistently observed between the DNAs of the two viruses (GOODHEART *et al.*, 1968; KIEFF *et al.*, 1971; GRAHAM *et al.*, 1972), suggesting differences in the nucleotide sequences of the two DNA molecules. Recent reassociation kinetic studies suggest that, unlike the DNA of HSV-1, about 16% of the DNA of HSV-2 consists of repetitive sequences (ROIZMAN and FRENKEL, 1973). DNA-DNA hybridization studies indicate that 50-70% of the base sequences of HSV-1 and HSV-2 DNA are homologous (KIEFF *et al.*, 1971; LUDWIG *et al.*, 1972). A difference appears to exist in the transcription of HSV-1 and HSV-2 DNA in that less HSV-2 DNA (21%) is transcribed early in infection (2 hours), prior to the onset of viral DNA synthesis, as compared with HSV-1 DNA (48%); about 50% of both viral DNAs is transcribed late (8 hr) in infection (ROIZMAN and FRENKEL, 1973). RNA-DNA hybridization studies indicate that 40-50% of the total HSV-1 DNA sequences transcribed is common to HSV-2 DNA (BRONSON *et al.*, 1972; ROIZMAN and FRENKEL, 1973). Intertypic complementation studies

with well defined ts mutants of HSV-1 and HSV-2 should shed further light as to the common genes of the two viruses.

It has been recently reported that cells from a human cervical carcinoma, free of infectious HSV-2, contain a DNA fragment with sequences corresponding to 39% of HSV-2 DNA and an RNA transcript complementary to 5% of viral DNA (FRENKEL *et al.*, 1972); this indicates a state of nonproductive HSV-2 infection in these cells in which only part of the viral genome is expressed, similar to the situation with cells transformed by known DNA tumor viruses. However, these findings do not prove that the HSV-2 genome is the primary cause of the cancer under study. Hamster cells transformed by HSV-2 and free of infectious virus also appear to contain only part of the genetic information of the virus used to transform them (RAPP and DUFF, 1973). The availability of well characterized ts mutants of HSV indeed offers a possibility to use them in cell transformation studies and to identify the viral gene functions required for transformation.

II. Temperature-Sensitive Mutants of HSV-1

Preliminary reports (SUBAK-SHARPE, 1969; SCHAFFER *et al.*, 1970) have described the isolation of ts mutants of HSV-1 following mutagenesis with 5-bromodeoxyuridine (BUDR). Since then we have isolated additional mutants following mutagenesis of wild-type virus with nitrosoaguanidine (NTG) and ultraviolet (UV) irradiation. Twenty-two of the mutants have been partially characterized and placed into complementation groups (SCHAFFER *et al.*, 1971; SCHAFFER *et al.*, 1973; ARON *et al.*, 1973).

The KOS strain of HSV-1 was used as WT virus. Isolation and characterization of mutants was carried out in human embryonic lung fibroblasts. Mutagenesis was carried out with WT virus that had been cloned 3 times at the permissive temperature and passed 3 times at the nonpermissive temperature to eliminate pre-existing ts mutants. Water-jacketed CO_2 incubators (5%) with temperature variations of $\pm 0.2\,^{\circ}C$ and constant-temperature water baths with temperature variations of $\pm 0.1^{\circ}$ were used for incubation of cells in petri dishes and closed vessels, respectively (SCHAFFER *et al.*, 1970). We had used initially (SCHAFFER *et al.*, 1970, 1971) 35° and 40° as the permissive and nonpermissive temperatures respectively. However, it was further found that at 40° efficient complementation experiments could not be carried out with the mutants in study. Thus 34° and 39° were adopted as the permissive and nonpermissive temperatures respectively; these conditions did not alter the initial observations reported (SCHAFFER *et al.*, 1970, 1971, 1973).

The procedures for WT virus mutagenesis with BUDR (0.3-5 μg/ml), NTG (20 μg/ml) and UV irradiation (30 or 60 sec., using a Sylvania

G15T8 bulb at a distance of 20 cm), and for mutant isolation have been described in detail elsewhere (SCHAFFER *et al.*, 1970, 1973). Sixty-one ts mutants of HSV-1 (48 BUDR-derived, 7 NTG-derived and 6 UV-derived) have been isolated to date, most of them at a mutagen dose reducing virus yields to about 1% of the control yield (SCHAFFER *et al.*, 1973). Twenty-two of these mutants have been studied in detail to date. The scheme proposed by ROBB *et al.* (1972) for the nomenclature of SV40 mutants has been partially adopted for the designation of HSV mutants (fig. 1). Each mutant name is composed of five components: (1) *Roman numeral I* to designate mutants of HSV type 1 (HSV type 2 mutants will be designated by *Roman numeral II);* (2) *ts,* temperature-sensitive;

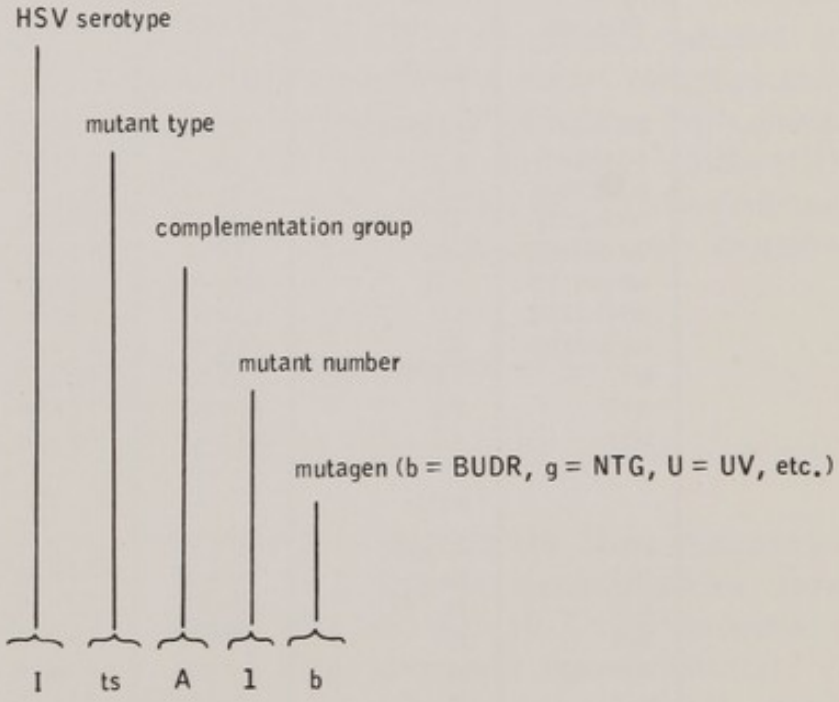

FIG. 1. Scheme for mutant nomenclature.

(3) *capital letters A, B, C,* etc., to designate complementation group; (4) *Arabic numbers 1, 2, 3,* etc., to designate mutant number (each number to be used only once); and (5) *lower case letters b, g and u,* to designate *mutagens BUDR, NTG and UV light, respectively* (SCHAFFER *et al.*, 1973).

A. Replicative Properties of Mutants

Comparative virus yields obtained at 34° and 39°, plating efficiencies (39°/34°), and leakiness and reversion at 39° of the 22 mutants studied are presented in table I. Eleven mutants were BUDR-induced, 7 were

NTG-induced and 4 were UV-induced. At 34°, yields of infectious mutant virus approached the yield of WT virus, while at 39°, yields of mutant virus were consistently at least 1000-fold less than the yield of WT virus. Plating efficiences, 39°/34°, ranged from 7×10^{-7} (ts18) to 1×10^{-4} (ts19 and ts20). All mutants exhibited some degree of leakiness and all but 5 mutants reverted with low frequency (table I). The highest reversion frequences

Table I.*

Virus	Mutagen and concentration or dose	Virus yield[b] (PFU/ml)		EOP[c] (39°/34°)
		34°	39°	
Wild-type	——	1.4×10^{8}	4.6×10^{7}	1
	BUDR:			
ts1 (ts343)[a]	1.25 μg/ml	1.2×10^{8}	1.5×10^{4}	3×10^{-5}
ts2 (ts847)	1.25 "	1.8×10^{7}	2.5×10^{3}	2×10^{-5}
ts3 (ts864)	2.5 "	8.0×10^{6}	2.1×10^{3}	2×10^{-5}
ts4 (ts867)	2.5 "	1.8×10^{7}	2.5×10^{2}	7×10^{-5}
ts5 (ts1102)	5.0 "	1.7×10^{7}	1.7×10^{3}	7×10^{-6}
ts6 (ts1112)	1.25 "	2.5×10^{7}	8.7×10^{3}	3×10^{-6}
ts7 (ts1136)	5.0 "	3.5×10^{7}	5.5×10^{2}	3×10^{-6}
ts8 (ts1178)	0.3 "	1.8×10^{7}	1.1×10^{3}	4×10^{-6}
ts9	1.25 "	1.0×10^{8}	4.6×10^{4}	5×10^{-6}[d]
ts10	2.5 "	1.5×10^{8}	3.5×10^{4}	8×10^{-5}
ts11	2.5 "	2.5×10^{7}	1.9×10^{4}	3×10^{-6}
	NTG:			
ts12	20 μg/ml	2.5×10^{7}	5.5×10^{2}	2×10^{-6}[d]
ts13	20 "	6.0×10^{6}	3.0×10^{3}	5×10^{-6}
ts14	20 "	1.2×10^{7}	1.5×10^{3}	1×10^{-5}[d]
ts15	20 "	7.5×10^{6}	1.0×10^{2}	2×10^{-6}
ts16	20 "	1.0×10^{7}	1.0×10^{2}	1×10^{-6}
ts17	20 "	1.8×10^{7}	3.5×10^{2}	3×10^{-6}
ts18	20 "	3.0×10^{7}	1.5×10^{3}	7×10^{-7}[d]
	UV irradiation:			
ts19	60 sec.	5.5×10^{7}	1.3×10^{3}	1×10^{-4}
ts20	30 "	3.0×10^{7}	1.5×10^{4}	1×10^{-4}
ts21	60 "	1.8×10^{7}	2.0×10^{3}	1×10^{-5}[d]
ts22	60 "	5.0×10^{7}	5.0×10^{3}	8×10^{-5}

[a] Previous designation (SCHAFFER *et al.*, 1970).

[b] Values represent the average of from 2-4 separate determinations.

[c] Virus stocks were grown at 34° and assayed at 34° and 39°. EOP= $\frac{\text{PFU/ml 39°}}{\text{PFU/ml 34°}}$; values represent the average of 2-4 determinations.

[d] Mutants which do not exhibit reversion. For these mutants:

$$\text{EOP} = \frac{\text{reversion of the highest virus dilution (per ml) exhibiting leak at 39°}}{\text{PFU/ml at 34°}}$$

*From SCHAFFER *et al.*, 1973.

were observed among UV-induced mutants. The fact that mutants exhibited little leakiness and low reversion frequencies made them particularly well suited for complementation analysis. The problem of reversion, although not significant until the fifth or sixth passage in many cases, has necessitated great care in the preparation of virus stocks. The preparation of multiple stocks using high dilutions of previous stocks known to contain few revertants in the undiluted state was found to be necessary.

B. Complementation Groups

Complementation tests were performed to determine whether the 22 mutants were defective in the same or different cistrons. Complementation was carried out at 39° fo 18 hr with cultures infected at multiplicities of 2.5 PFU/cell of each mutant in a mixed infection and 5.0 PFU/cell of each mutant in single infections. Yields from both single and mixed infections were assayed at 34°. Complementation indices (CI) were determined by dividing the 39°-yield produced in mixed infections ($A \times B_{39}{}^{\circ}$) by the sum of the 39° yields produced by two mutants ($A_{39}{}^{\circ} + B_{39}{}^{\circ}$) grown separately

$$CI = \frac{(A \times B)_{39}{}^{\circ}}{A_{39}{}^{\circ} + B_{39}{}^{\circ}} \text{ when yields assayed at } 34°$$

Values of 2 or greater were taken as indicative of complementation (SCHAFFER *et al.*, 1973).

All possible pair-wise crosses were made with the 22 ts mutants in 2-5 replicate tests (table II). Four or 5 separate determinations were carried out using pairs whose indices were less than 10. Complementation indices ranged from less than 1 (ts5 × ts6) to greater than 9,000 (ts13 × ts18). Mutants failed to complement each other in only 8 of 231 cases. Although the complementation index is primarily a measure of whether complementation occurred or not, and only a partial measure of the efficiency of complementation, certain mutants in the set were characterized by generally lower indices (e.g., ts16) and others by higher indices (e.g., ts19). The efficiency of complementation with most mutant pairs was high; however, other pairs consistently complemented each other with low efficiency. Possible explanations for low efficiencies include the following: (a) instability or nondiffusability of gene products; (b) the possible inclusion of double mutants, not yet identified as such, in the series of mutants; (c) interference by one mutant with growth of the other at the nonpermissive temperature; and (d) intracistronic complementation as seen in the T4-*Escherichia coli* system in which complementation efficiencies similar to those found in the HSV system occur (EDGAR *et al.*, 1964).

*Table II**. Complementation between ts mutants of HSV type 1.

ts mutant	Yield from single infection[a] (PFU/ml)	Complementation index[b] from mixed infection with ts mutant:																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
ts1	1.2×10^{3}	–	570	180	2250	1350	150	850	160	170	140	370	11	130	26	[1.2]	[0.7]	25	62	750	410	250	270
ts2	2.5×10^{2}	–	–	1400	2050	1900	350	1250	41	850	43	110	6.9	88	3.6	15	4.1	33	67	910	75	[1.3]	370
ts3	3.3×10^{2}	–	–	–	180	570	280	180	[1.0]	52	68	120	12	140	11	9.4	2.9	49	53	250	86	280	150
ts4	5.2×10^{1}	–	–	–	–	1100	750	[0.9]	42	76	160	130	61	530	160	190	18	470	1350	950	270	1250	52
ts5	6.5×10^{1}	–	–	–	–	–	[0.5]	500	120	570	48	150	13	150	93	54	4.7	48	120	610	50	670	13
ts6	2.6×10^{2}	–	–	–	–	–	–	430	400	1350	54	160	10	100	31	26	4.5	42	170	1100	15	950	20
ts7	3.3×10^{2}	–	–	–	–	–	–	–	160	70	91	160	46	32	35	12	6.6	390	780	350	130	470	6.4
ts8	3.1×10^{2}	–	–	–	–	–	–	–	–	100	10	21	5.4	21	18	3.8	4.1	6.9	54	41	16	1150	4.8
ts9	7.4×10^{2}	–	–	–	–	–	–	–	–	–	31	95	23	47	16	35	14	43	55	2250	190	110	2.7
ts10	1.4×10^{3}	–	–	–	–	–	–	–	–	–	–	2.9	27	28	15	140	450	280	190	700	35	94	150
ts11	3.7×10^{3}	–	–	–	–	–	–	–	–	–	–	–	25	100	53	38	3.2	27	200	510	200	1300	150
ts12	1.3×10^{3}	–	–	–	–	–	–	–	–	–	–	–	–	3400	230	20	68	350	300	580	550	7.4	.160
ts13	5.6×10^{2}	–	–	–	–	–	–	–	–	–	–	–	–	–	5100	1950	12	970	9300	3.9	4250	640	1150
ts14	5.3×10^{2}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	19	6.3	24	173	1150	530	7.2	290
ts15	4.4×10^{2}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	[0.8]	170	950	1500	390	13	300
ts16	5.0×10^{2}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	40	170	58	580	1250	660
ts17	2.3×10^{3}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	[1.4]	2500	1200	60	620
ts18	4.3×10^{2}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5050	1700	770	4300
ts19	1.2×10^{3}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	970	450	1700
ts20	5.1×10^{3}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	230	5.1
ts21	1.1×10^{1}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	420
ts22	5.6×10^{2}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Yields from single infections are the averages of all tests using each mutant.

[b] Complementation index $= \frac{(A\times B)_{39°}}{A_{39°}+B_{39°}}$ Assayed at 34°. Values less than 2 (numbers in boxes) were considered to be negative.

*From SCHAFFER *et al.*, 1973.

*Table III**. Complementation groups of HSV type 1.

Complementation group	Mutants	Viral DNA phenotype 39°
A	ts1 (I ts A 1 b)[a] ts 15 (I ts A 15g) ts16 (I ts A 16 g)	−
B	ts2 (I ts B 2 b) ts21 (I ts B 21 u)	−
C	ts4 (I ts C 4 b) ts7 (I ts C 7 b)	−
D	ts9 (I ts D 9 b)	−
E	ts5 (I ts E 5 b) ts6 (I ts E 6 b)	+
F	ts17 (I ts F 17 g) ts18 (I ts F 18 g)	+
G	ts3 (I ts G 3 b) ts8 (I ts G 8 b)	+
H	ts10 (I ts H 10 b)	+
I	ts11 (I ts I 11 b)	+
J	ts12 (I ts J 12 g)	+
K	ts13 (I ts K 13 g)	+
L	ts14 (I ts L 14 g)	+
M	ts19 (I ts M 19 u)	+
N	ts20 (I ts N 20 u)	+
O	ts22 (I ts O 22 u)	+

[a] Complete mutant designations as described in fig. 1.
* From SCHAFFER *et al.*, 1973.

By the complementation criteria described above, the 22 mutants tested fell into 15 non-overlapping complementation groups (table III). It is evident from the results of the complementation studies just described that the three mutagenic agents used can cause defects in the same viral function, as exemplified by complementation groups A and B, which contain mutants induced by different agents (tables I and III). Whether highly mutable cistrons exist for HSV as found for vesicular stomatitis virus (PRINGLE, 1970) cannot be determined accurately until additional mutants are isolated and placed into functional complementation groups.

C. Partial Characterization of Mutants

1. Viral DNA Synthesis. The viral DNA phenotypes of the mutants grown at the nonpermissive temperature were determined by equilibrium sedimentation (44,000 rev/min, 20 hr at 25°) in neutral CsCl of the DNA from infected-cell lysates, using a Spinco model E ultracentrifuge (SCHAFFER *et al.*, 1973). Viral DNA (ρCsCl= 1.725 gm/cm^3) was readily detectable in analytical ultracentrifuge tracings of lysates of cells infected with WT virus and maintained at either 34° or 39° as well as in lysates of cells infected with each of the 22 mutants at 34°. Fourteen mutants in complementation groups E through O synthesized significant amounts of viral DNA at 39° and were considered to have a DNA$^+$ phenotype (table III).

No viral DNA was detected in lysates of cultures infected at 39° with the remaining 8 mutants in complementation groups A through D. Thus, 5 of 11 (43%) BUDR-induced mutants, 2 of 7 (29%) NTG-induced mutants, and 1 of 4 (25%) UV-induced mutants were viral DNA$^-$ at 39°. The occurrence of 4 complementation groups whose members exhibit DNA$^-$ phenotypes at 39° suggests that at least 4 viral genes are concerned with HSV DNA synthesis, assuming that each complementation group represents a viral cistron. The method used to screen mutants for their ability to synthesize viral DNA may not have detected small amounts of DNA synthesized by some mutants. However, each determination was repeated either 2 times (DNA$^+$ mutants) or 4 times (DNA$^-$ mutants). In addition, DNA$^-$ mutants always complemented DNA$^+$ mutants (table III). More precise quantitation of mutant-induced DNA synthesis using isotopic labeling techniques is currently in progress.

2. Thermal Stability. Preliminary attempts were made to determine whether any of the 22 mutants was defective in a structural component of the mutant virion rendering it more thermolabile. Thermal stabilities of the 22 mutants and of the WT virus were tested at 39° (fig. 2A) and 8 BUDR-induced mutants were also tested at 45° (fig. 2B). These results represent the average of two separate determinations. Because of the rapid inactivation of all viruses at 45°, no marked differences were observed between the thermal stability of the WT virus and the 8 mutants tested at this temperature. Although some of the 22 mutants exhibited greater thermal stability that the WT virus at 39° (e.g., ts10, 19 and 17), other mutants were significantly less stable at this temperature (e.g., ts3, 6, 5, 4 and 8). Although the precise nature of the ts defects of the mutants is not yet known, the demonstration that certain mutants exhibited altered patterns of thermal stability at 39° compared with the WT virus suggests that the ts defects of these mutants may involve structural components of the virion. It is of interest that within complementation groups E and G, individual members (ts 5 and 6 and ts3 and 8, respectively) behaved

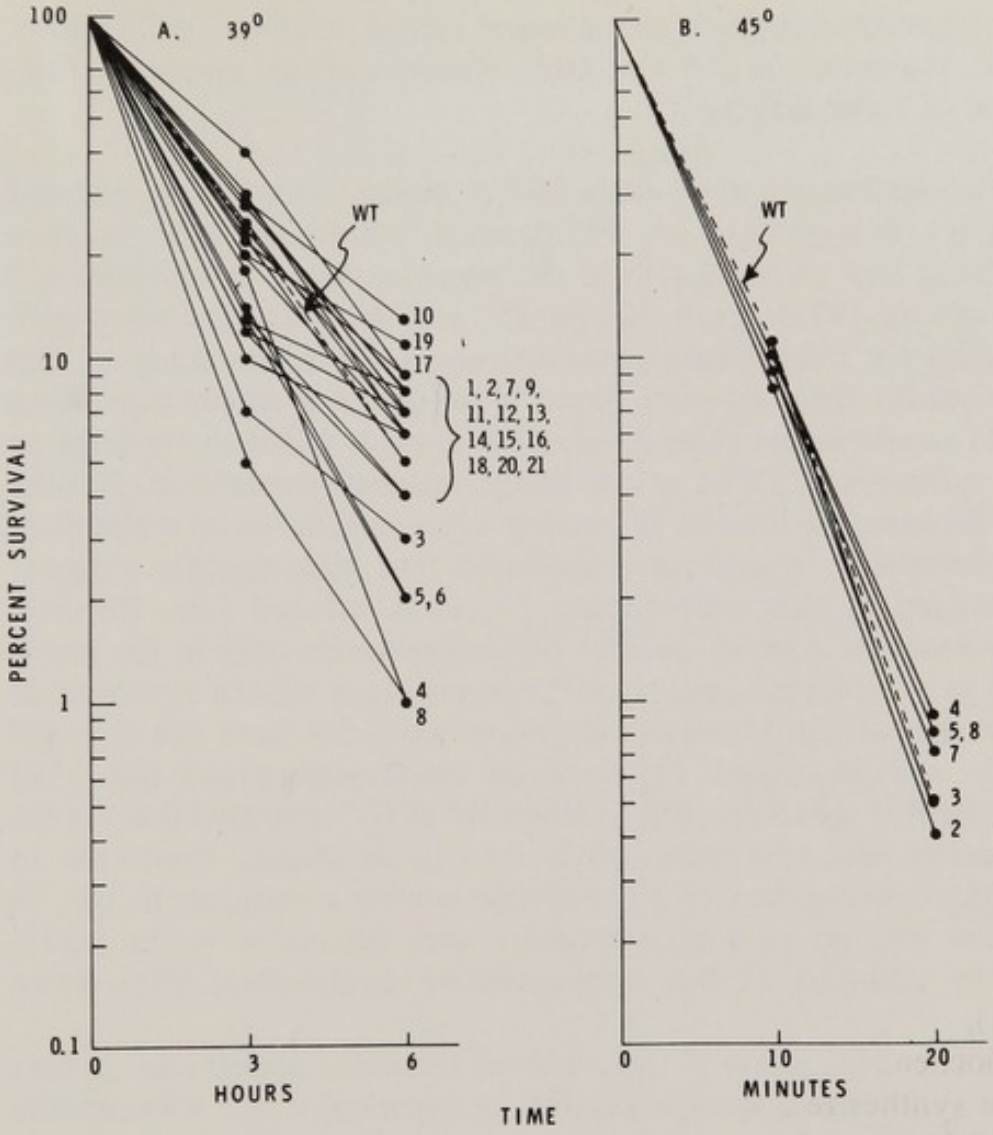

FIG. 2. Thermal inactivation of wild-type HSV-1 and ts mutants. Cell-free virus preparations were diluted 100-fold in Tris (to obtain a final serum concentration of 0.05%) and then passed through 450-mμ Millipore filters to remove virus aggregates. The preparation were heat-treated at 39° (A) and 45° (B) and at the times indicated duplicate samples were assayed for residual infectivity. Results are presented as percent virus survival compared with unheated, control preparation 100%). ----, wild-type virus; ———, ts mutants (From SCHAFFER *et al.*, 1973).

similarly to each other with regard to patterns of thermal inactivation at 39°.

3. Protein and Glycoprotein Synthesis. To determine further the biochemical defects of the mutants in study, a detailed SDS-polyacrylamide gel electrophoretic (PAGE) analysis of polypeptides and glycopeptides synthesized at the permissive and nonpermissive temperatures is being carried out. Preliminary evidence suggests that mots of the DNA^+ mutants are capable of inducing the synthesis of all the major viral peptides at the nonpermissive temperature whereas some of the DNA^- mutants appear

to be defective in the synthesis of the major capsid polypeptide C4 (BONE *et al.*, 1973). However, further and more precise analysis awaits the final identification of these defects.

Of particular interest to us was a DNA$^-$ mutant, ts343 (now assigned the number ts1, SCHAFFER *et al.*, 1973), which was found to be defective in the synthesis and glycosylation of the major envelope polypeptide C5 (SCHAFFER *et al.*, 1971). Even though 35° and 40° were the permissive and nonpermissive temperatures, respectively, used in the study of this mutant, the results reported were found to hold true in repeat tests using 34° and 39° temperatures respectively. The mutant failed to replicate or induce the synthesis of DNA at the nonpermissive temperature. Results of immunofluorescence studies of mutant virus-infected cells maintained at the nonpermissive temperature indicated that virus-specific antigens were synthesized at this temperature, but at a reduced rate. Electron microscopy revealed a small number of nucleocapsids only in the nuclei of 2-5% of ts343-infected cells at 40°, suggesting a limited synthesis of late functional proteins. However, enveloped particles were not detected in any of the cells examined. Furthermore, electropherograms (fig. 3) of isotopically-labeled ts343 proteins synthesized at 40° indicated that the C5 envelope protein was not being synthesized in significant quantities. In addition, electropherograms of glucosamine-labeled glycoproteins (fig. 3) also indicated that no viral glycoproteins were detectable in the ts343-infected cells cultured at the nonpermissive temperature (SCHAFFER *et al.*, 1971).

The biochemical lesion of this mutant therefore appears to involve its failure to synthesize a specific protein (or proteins) which is eventually glycosylated by transferase enzymes. It is also possible that normal proteins are synthesized to some extent but that the transferase enzymes required for glycosylation do not function properly at the elevated temperature . The data obtained appear to rule out the second hypothesis in that normal glycosylation of the WT virus proteins occurred at 40° as well as at 35°, and thus the necessary transferase enzymes seem not to be affected by the elevated temperature. This observation would be further verified if the transferase enzymes in HSV-infected cells are found to be host-specified, as has been suggested for Sindbis virus-infected cultures (GRIMES and BURGE, 1971).

The absence of significant quantities of protein C5 as well as glycosylated viral macromolecules may well be a secondary effect resulting from an as yet undetectable primary biochemical lesion in this ts mutant. The synthesis and glycosylation of detectable quantities of protein C5 may be directly dependent on viral DNA synthesis or on some other early biochemical event within the virus-infected cell. Further biochemical as well as immunochemical characterization of this mutant and other ts

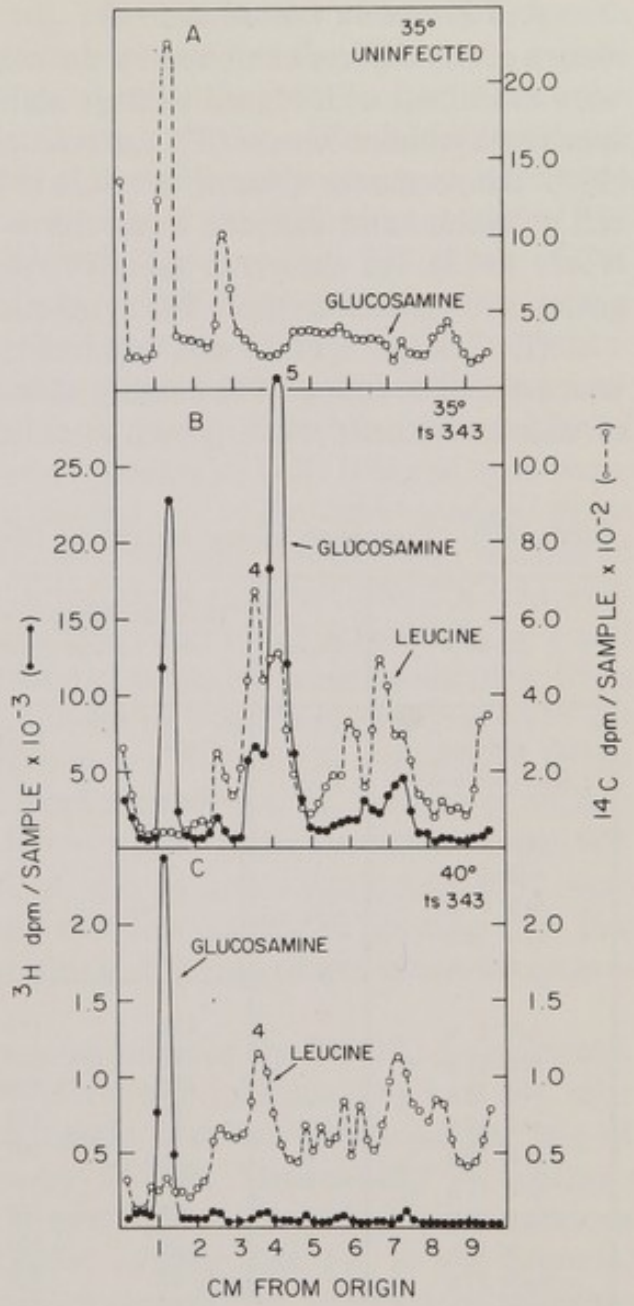

FIG. 3. Acrylamide gel electropherogram of the cytoplasmic glycoproteins from HEL cells infected with ts343 and maintained at 35° or 40°. Cytoplasmic fractions were obtained from cultures isotopically labeled for 4-24 hr after infection. Samples electrophoresed were: (A) glucosamine-^{14}C (0.01 μCi/ml)-labeled uninfected control culture, 35°; (B) doubly labeled glucosamine-^{3}H (10 μCi/ml) and leucine-^{14}C (0.1 μCi/ml) cultures infected with ts 343, 35°; (C) same as B but at 40°. dpm = disintegrations per min. (From SCHAFFER *et al.*, 1971).

mutants belonging to different complementation groups should provide a more precise definition of the biochemical lesion of this particular mutant. However, mutants of this type should be useful to delineate further the precise roles that the host cell and the virus play in determining the carbohydrate sequence of HSV-induced glycopeptides.

4. Thymidine kinase activity. In further attempts to determine the nature of the defects exhibited by the ts mutants, 18 representative mutants were examined with regard to their ability to induce the synthesis of HSV-specific thymidine kinase (TK) at both permissive (34°) and nonpermissive (39°) temperatures (ARON *et al.*, 1973). The detailed procedures for cell infection and enzyme assay have been described elsewhere (ARON *et al.*, 1973). As shown in table IV, the mutants studied fell into 3 categories with regard to their TK synthetic abilities at 34° and 39°:

(I) Group I (TK^-) consisted of 5 mutants, all induced by BUDR, which failed to induce TK activity at levels significantly greater than those of uninfected cells when grown at either 34° or 39°.

*Table IV**. Thymidine kinase activity of HSV type 1 temperature-sensitive mutants.

Virus	Group	Thymidine kinase activity[b] when virus grown at: 34°	39°	DNA phenotype[c] 39°
HSV wild-type		(100)	(100)	+
Control-uninfected		5	5	
ts A 1 b[a]	I (TK^-)	4	4	–
ts B 2 b		5	5	–
ts E 6 b		4	5	+
ts – 24 b		5	5	+
ts G 3 b		10	6	+
ts C 7 b	II (TK^+)	112	113	–
ts A 15 g		95	74	–
ts A 16 g		125	129	–
ts J 12 g		77	102	+
ts K 13 g		57	103	+
ts F 17 g		95	122	+
ts F 18 g		101	103	+
ts M 19 u		70	127	+
ts N 21 u		83	106	+
ts O 22 u		103	115	+
ts – 23 b	III ($TK^\pm$)	115	24	–
ts L 14 g		100	45	+
ts B 20 u		81	25	+

[a]Mutant designation: capital letters designate complementation group (dashes indicate mutants not assigned as yet to complementation groups), Arabic numerals designate mutant number and lower case letters designate mutagens (b= 5BUDR, g= NTG, u= UV-irradiation).

[b]Cultures were infected at a MOI of 5 PFU/cell and incubated at either 34° or 39° for 10 hr. Cell extracts were prepared and TK activity was assayed using deoxyuridine-6-H^3 (UDR-6-H^3, 13.3 Ci/mM; New England Nuclear Corp., Boston) as nucleotide acceptor as described (KIT *et al.*, 1963; KIT and DUBBS, 1965). Results are expressed as percent wild-type picomoles dUMP formed/μg protein in 10 min at 39° and values given represent averages of 2-5 separate determinations.

[c](See SCHAFFER *et al.*, 1973).

*From ARON *et al.*, 1973.

(II) Group II (TK^+) consisted of 10 mutants which, in general, induced kinase activity at levels similar to those of the WT virus at both temperatures. These mutants were not studied further.

(III) Group III ($TK^\pm$) consisted of 3 mutants which differed from the first two groups in that the levels of kinase activity observed in infected cultures grown at 34° were similar to that of the WT virus, while at 39° kinase activity was significantly reduced. The fact that WT levels of TK activity could be detected in 39° assays when these mutants were grown at the permissive temperature suggested that the TK was not temperature-sensitive *per se* but rather, that a step in the production of TK was temperature-sensitive. To verify this assumption, heat-inactivation kinetic studies were carried out with enzyme extracts of cells infected with these 3 mutants at 34°. Inactivation was carried out *in the absence of substrate* at 39°, 40°, and 45°, respectively, and residual activity was compared with that of WT enzyme treated in the same manner. The TK enzymes induced by two of the $TK^\pm$ mutants described in table IV (ts L 14 g and ts B 20 u) revealed thermoinactivation patterns indistinguishable from that of WT virus-induced enzyme, indicating that these enzymes were not temperature-sensitive but the mutant defect was in TK production at the nonpermissive temperature. On the other hand, the TK activity of mutant ts-23 b (table IV) was significantly more heat-labile than that of the WT enzyme at both 39° and 40° (at 45° all enzymes, including WT, were inactivated at approximately equal and fast rates), suggesting that this mutant indeed induces a kinase which is temperature-sensitive (Aron *et al.*, 1973).

The finding that of the 7 ts mutants induced by BUDR, 5 exhibited a TK^- defect at both 34° and 39° (Group I in table IV) and are thus independent of the ts defect, is consistent with the notion on the loss of TK gene function from selection by BUDR. Their ability to replicate at 34° in the absence of viral TK synthesis is consistent with previous observations (Kit and Dubbs, 1963a, 1963b) that TK is not essential for virus replication. However as discussed earlier, herpesvirus infection results in the appearance of TK with new physicochemical properties from those of pre-existing cell enzymes (Klemperer *et al.*, 1967; Kit *et al.*, 1967). We thus used the mutants which were TK^- at 34° and 39° in an attempt to distinguish between cellular and viral TK activity by non-SDS-polyacrylamide gel electrophoresis (Aron *et al.*, 1973).

Representative results with TK induced by WT virus, mutants ts E 6 b (TK^- at both 34° and 39°), and mutants ts−23 b ($TK^\pm$) are presented in figure 4. Gels of native extracts from uninfected and WT-infected cells maintained at 39° showed different electrophoretic patterns (fig. 4A). Cell TK migrated as one major species (C_1) approximately 5 cm (Rf= 0.5) from the origin and one minor species (C_2) approximately 2 cm (Rf= 0.21) from the origin. The WT virus induced two addi-

tional TK species when compared with cell TK patterns: a major species (V_1) approximately 4 cm (Rf= 0.41) from the origin and a minor species (V_2) approximately 3 cm (Rf= 0.32) from the origin (fig. 4A). Patterns of TK activity from extracts of infected and control cells grown at 34° are not shown but were similar to their 39° counteparts. PAGE profiles

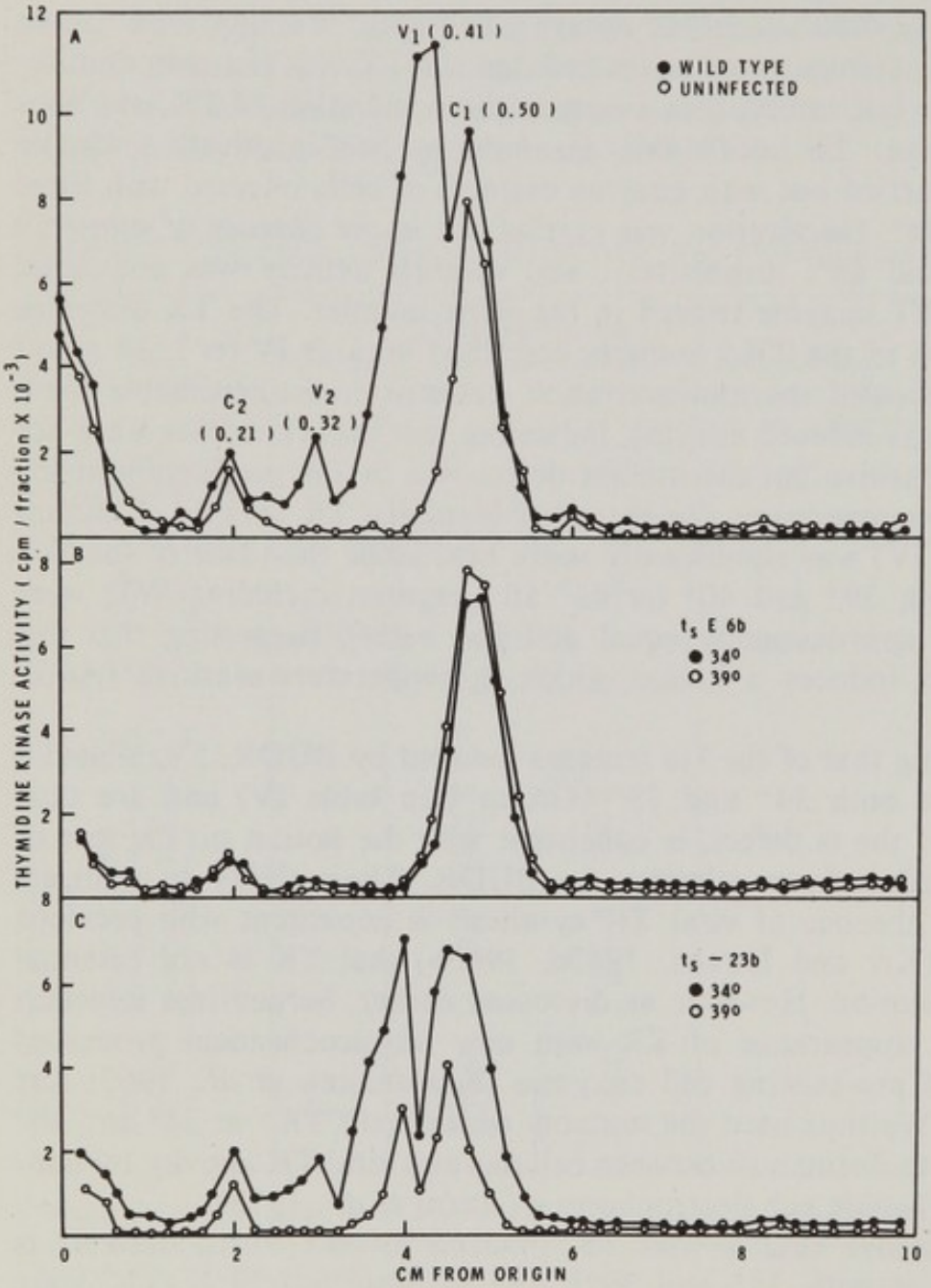

FIG. 4. Polyacrylamide gel electrophoresis of enzyme extracts. Uninfected and infected (MOI= 5 PFU/cell) cells were harvested after 10 hr incubation at either 34° or 39°. Enzyme extracts were electrophoresed as described by DAVIS (1964) but in the absence of sodium dodecyl sulfate. TK activity in the gels was determined by the "sectioning" method essentially as described by MUNYON *et al.* (1972) only the gel slices (2mm) were incubated for 5 hr at 39° instead of 4 hr at 37° (From ARON *et al.*, 1973).

of enzyme extracted from cells infected with the TK^- mutant (ts E 6 b) at both temperatures exhibited peaks of activity, corresponding to cell TK only (fig. 4B), confirming the inability of TK^- mutants to induce functional viral TK. The enzyme synthesized by the Group III ($TK^{\pm}$) mutant ts−23 b at 34° revealed a gel pattern similar to that of the enzyme synthesized by the WT virus (fig. 4C). However, in extracts prepared from cells infected with the $TK^{\pm}$ mutant at 39°, peak V_1 was greatly reduced and peak V_2 was not detected and this was consistent with the previously demonstrated (table IV) depression in TK activity at 39° in mutants belonging to Group III.

The evidence that (1) infection with $TK^{\pm}$ mutants results in a substantial reduction of TK activity under nonpermissive conditions as compared to permissive conditions, and (2) the TK induced by mutant ts−23 b at the permissive temperature has an increased sensitivity to heat at the nonpermissive temperature, suggests that herpesvirus codes for a new TK. The gel patterns presented in figure 4 indicated that the increase in TK activity following herpesvirus infection is due to the appearance of additional specie(s) of TK activity differing from cell TK in charge, molecular weight, and/or configuration, or in any combination of these factors. These results are in good accord with the data of MUNYON *et al.* who studied cells that had been transformed from a TK^- to a TK^+ phenotype by UV-inactivated HSV and found that the new TK enzyme acquired by the HSV transformed cells migrates to the same location in PAGE as does TK activity induced in TK-negative L cells (Ltk minus) during a lytic infection by HSV (MUNYON *et al.*, 1971, 1972).

D. Recombination

We have reported earlier (SCHAFFER *et al.*, 1972) that efficient recombination occurs in two-factor crosses between ts mutants of HSV-1. Thus far, 14 mutants belonging to 9 complementation groups (A, B, E, F, G, J, M, N and O presented in table III) have been examined for their ability to recombine at the permissive (34°) temperature (SCHAFFER and BENYESH-MELNICK, 1973). Cells were infected in the same manner used for complementation (SCHAFFER *et al.*, 1973). Mixedly infected cultures (2.5 PFU/cell of each mutant) and singly infected cultures (5 PFU/cell) were incubated at 34° for 18 hr and the virus yields assayed at 34° and 39°. Recombination frequency was calculated by the equation

$$\frac{(A \times B)_{34^\circ}, \text{ assayed at } 39^\circ}{(A \times B)_{34^\circ}, \text{ assayed at } 34^\circ} \times 2 \times 100, \text{ where}$$

A is one mutant and B is the other (SCHAFFER and BENYESH-MELNICK, 1973). Results based on 2-5 determinations obtained to date of each

pairwise cross are given in table V. Noncomplementing mutant pairs either failed to recombine (ts 5×ts 6, ts 17×ts 18, ts 3×ts 8) or recombined with low frequency (ts 15×ts 16, ts 2×ts 21). No recombination could be detected between complementing mutants ts 5 and ts 19. The remaining crosses yielded recombination frequencies ranging from 2% to 52%, suggesting efficient exchange of genetic material during mixed infection.

Table V. Recombination* between ts mutants of HSV type 1.

		15	16	2	21	5	6	17	18	3	8	12	19	20	22
A**	ts 15g	–	3	NT†	NT	NT	NT	16	12	NT	NT	13	NT	NT	NT
	ts 16g		–	NT	17	NT	NT	15	10	NT	NT	9	8	NT	5
B.	ts 2b			–	4	16	21	24	18	21	25	45	17	42	34
	ts 21u				–	20	21	31	24	56	25	10	33	31	23
E	ts5b					–	0	26	12	4	8	12	0	10	5
	ts6b						–	10	8	13	5	20	9	52	9
F	ts 17g							–	0	10	20	10	2	3	7
	ts 18g								–	7	29	11	4	4	5
G	ts 3b									–	0	7	23	19	6
	ts 8b										–	10	5	10	7
J	ts 12g											–	5	8	15
M	ts 19u												–	40	29
N	ts 20u													–	6
O	ts 22u														–

*Recombination frequency= $\frac{(A \times B)_{34^\circ}, \text{ assayed at } 39^\circ}{(A \times B)_{34^\circ}, \text{ assayed at } 34^\circ}$

**Complementation groups (see table III).
† Not tested.

Clumps of complementing mutant virions or complementing heterozygotes can mimic WT recombinants when mixed yields are assayed at the nonpermissive temperature. Subjecting mixed yield of complementing mutant pairs to sonication and/or filtration failed to alter the percent of progeny yielding plaques at 39° (WT recombinants). Furthermore progeny testing was carried out with the mixed yields of 5 mutant pairs (ts 2×ts 12, ts 12×ts 3, ts 12×ts 6, ts 17×ts 22, and ts 8×ts 18). The mixed yield of each pair was plated at 39° and a total of 55 well-isolated plaques were picked and assayed for infectivity at 34° and 39°. Fifty-four of the 55 plaque isolates had the same efficiency of plating at 34°

and 39° and thus behaved as true recombinants (SCHAFFER and BENYESH-MELNICK, 1973). Further progeny testing is being done; however it is apparent from these results that in most of the cases we are dealing with true WT recombinants.

The data indicate thus far that efficient recombination can occur between ts mutants of HSV-1, at relatively high frequency. As indicated earlier the relatively large size of the double-stranded DNA of HSV (100×10^6 daltons) provides an ideal molecule for genetic exchange. It remains to be seen whether the non-random single-strand nicks in HSV-DNA (FRENKEL and ROIZMAN, 1972) are prefered sites for breakage and reunion. The finding that noncomplementing mutants either failed to recombine or recombined with low frequency are confirmatory of the complementation data and demonstrate that mutants defective in the same gene do not recombine efficiently.

Based on the data obtained thus far, a provisional sequencing of mutants representing 7 complementation groups in the order of: B (ts2, ts21) – E (ts5, ts6) – G (ts3, ts8) – O (ts22) – F (ts17, ts18) – N (ts 20) – J (ts 12) has been obtained (SCHAFFER and BENYESH-MELNICK, 1973). Even though preliminary, this 7 gene map indicates that (I) ts mutants of HSV-1 can be additively mapped using recombination data from two-factor crosses, (II) the series of mutants studied contain individual mutants at both ends of the theoretical 50-unit map, and (III) the map is so far linear and not circular. The ordering of further mutants is necessary to verify these preliminary conclusions. Furthermore, mutants with additional markers permitting three-factor crosses should be of advantage.

Using BUDR-derived ts mutants of a syncytial strain (Glasgow strain 17) of HSV-1, SUBAK-SHARPE's group has reported that 9 independent mutants fell into 8 complementation groups. Using three-factor crosses a provisional 8 gene map has been proposed (S. M. BROWN, D. A. RITCHIE and J. H. SUBAK-SHARPE, cited in HAY *et al.*, 1971; SUBAK-SHARPE, 1973).

III. Temperature-Sensitive Mutants of HSV-2

In 1971 the Glasgow group reported the isolation of 33 stable ts mutants of HSV-2 (strain HSG 52) after mutagenesis with BUDR (5 μg/ml) in BHK21, cone 13 cells (TIMBURY, 1971). The efficiency of plating (EOP) of all mutants was low at the nonpermissive temperature (38°) as compared to the permissive temperature (31°)—EOP 38°/31° values ranging from 2.2×10^{-4} to $< 9.1 \times 10^{-8}$. Complementation tests could not be carried out with virus yields from monolayer cultures mixedly

infected and singly infected with each mutant. Successful complementation was attained with the use of an infectious center assay even though variable results, attributable to low yields of virus, were noted. Ten of the mutants were placed into 10 different complementation groups. More recently, it was reported (SUBAK-SHARPE, 1973) that certain ts mutants of HSV-2 and of HSV-1 complemented each other in mixed infections.

We have thus far isolated 11 ts mutants of HSV-2 (strain 186) after mutagenesis with BUDR following the same procedures described for derivation of HSV-1 ts mutants, except that 34° and 38° were used as the permissive and nonpermissive temperature, respectively (ESPARZA *et al.*, 1973). Work with 8 of these mutants has indicated the following: (1) The mutants are stable and yield EOP 38°/34° values ranging between 1.8×10^{-4} and 3.0×10^{-7}. (2) Complementation tests can be carried out exactly as with the HSV-1 ts mutants (except for the use of 38° as the nonpermissive temperature)—and thus far the 8 mutants fall into 7 nonoverlapping complementation groups. (3) Members of 3 of these groups are of DNA^- phenotype at the nonpermissive temperature, the remaining being DNA^+. (4) Intertypic complementation could be demonstrated with several of the HSV-2 ts mutants and ts mutants of HSV-1; noncomplementing pairs of HSV-1 mutants either both complemented certain HSV-2 mutants or both failed to complement other HSV-2 mutants (ESPARZA *et al.*, 1973). Once substantiated, these latter findings offer a good possibility for the identification of common gene functions between the two strains of HSV.

In conclusion, it is obvious from the data presented here that genetic work with HSV is indeed in its incipient stages. However, the results obtained to date indicate that ts mutants of HSV are beginning to provide a powerful tool for genetic analysis of viral functions, and hopefully for those functions that may be concerned with transforming a cell into the neoplastic state.

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VIRAL ONCOGENESIS AND CHEMICAL CONTROL*

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CONTENTS

Viral Oncogenesis and Chemical control*

I. Introduction

It may be useful to introduce the system in which we are attempting to study the process of the conversion, or transformation, of living cells into a condition which simulates, or models, the cancerous one. It has been known for about 50 years. ROUS was the first to clearly demonstrate that it was possible to produce a tumor in the chickens by the use of a clean virus preparation. It took almost 40 years before it was recognized that this experiment constituted the beginning of a really important era in virology and in oncology. Following this, about a dozen years ago, it became possible to count viruses (virions) by a technique called focus formation. This technique resembles very much the phage assay technique. In this, a gel containing the uniform bacterial inoculum is first prepared. A virus assay is made by taking a dilute aliquot of the virus suspension, infecting the bacterial plate, and wherever a single virus infects a bacterium, that bacterium will eventually lyse, liberate more virus (phage) particles, resulting in a small clear spot in which would otherwise be a crowded plate uniformly clouded with a bacterial population. This results in the ability to actually count the phage particles. In the case of animal viruses, a related technique is used, particularly with the oncogenic viruses. In this case, the overall plan is very much the same, but instead of a poor plate of bacterial culture which looks cloudy and where the clear spots are counted, the technique is quite the reverse. A smooth, uniform layer of animal cells is infected with an aliquot of the virus which is being

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assayed, and allowed to grow from 3-7 days. Then, those cells which have been infected by the virus and which are transformed by the virus show, not as clear spots in a uniform background, but as highlights, because the cells which have been infected by the oncogenic virus and transformed grow rapidly and build up in little mounds which can be seen as high, dense cell populations instead of a single monolayer of cells. Figures 1 and 2 will show this phenomenon. The original use of the technique of focus formation was to count the oncogenic virions, just as the bacterial plate was used to count phage particles. In recent years the system has been turned around: not only is it used to count virions, but the idea that these foci might be considered as models of a cancer system has gained some significance; this is the kind of a system which I will be discussing.

Figure 1 shows cells in tissue culture through an optical microscope, showing the nucleus of each cell, with the cell membrane around the outer edge. You can see that the cells are in some kind of communication with each other; you can see the strands of contact between them. Actually, this picture shows the beginning of a growth on the plate; when the plate is completely filled, the cells will be in contact all around, on all sides of their cytoplasmic membrane, and growth will stop when the cells reach that particular stage of confluence in which they are in contact with each other. At that time apparently some "signals" are passed between them which stop the cell growth. This is a model for normal growth by normal cells. When the cells are infected by oncogenic viruses, either RNA or DNA viruses, the signaling system is somehow broken down, and the cells do not cease growing when they reach confluence. They overgrow each other, and the "foci" develop, as shown in figure 2. The piles of cells (foci), which might be taken to represent models of tumors in the whole animal, appear as light-reflecting piles on the plate; on the right-hand side of the figure it is possible to see individual cells piling on top of each other. The foci can be counted, each one representing a virus infection which has transformed a cell which then grows into a group of cells failing to show contact inhibition. In a sense, this tissue culture represents a model for the whole animal, and the focus represents the cancer.

What type of viruses are we talking about? We use oncogenic viruses, some DNA, and some RNA viruses. A common DNA virus which infects mammalian cells is SV40, the simian virus No. 40, which will infect and transform tissue cultures of monkey cells. A common RNA virus is murine sarcoma virus (MSV), which, in turn, infects and transforms a mouse tissue culture. We will be concerned almost exclusively with the RNA type viruses, although some of the discussion will be relevant to the DNA viruses as well.

Figure 3 shows an electron micrograph of a section of a special

FIG. 1. Nontransformed Balb/3T3 cells, 5500 x, showing contact between cells (optical microscope).

FIG. 2. MSV focus on Balb/3T3 cells left at 10 x magnification, right at 40 x (optical microscope, dark field illumination).

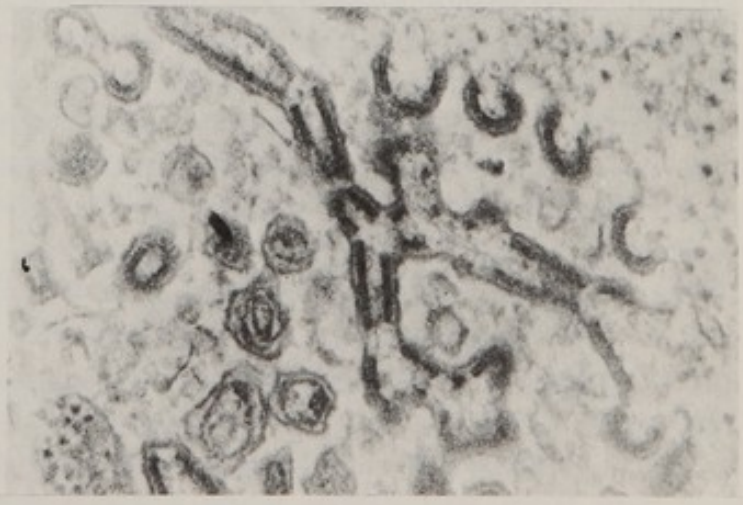


FIG. 3. Section through UC1-B cells showing both normal (MLV) and aberrant (MSV) Type C virus in cells. Left-hand side, complete MLV, C type; center, aberrant MSV, cylindrical and incomplete (magnification 240,000 x) (electron microscope).

strain of Balb cells infected with murine leukemia virus (MLV). The more standard type viruses are shown on the left-hand side of the figure. These are the Type C viruses, with the nucleoid in the center and capsid around the outside, representing almost complete murine leukemia viruses. In the upper right-hand corner of figure 3 are visible four invaginations of the vacuolar membrane showing the initiation of the encapsulation of the Type C viruses which ultimately close to give what appears to be a cellular membrane coated virion, mentioned earlier. This particular strain of cells, called Balb UC1-B, is a variant of the ordinary strains and is subject to transformation by MLV alone. We will speak more of the significance of this fact later.

It is believed that the outside membrane of the virus has many of the same qualities as the cell membrane itself. Finally, we believe that the elongated peculiar shapes in the center of figure 3 are the unfinished or aberrant sarcoma virus particles in this same cell. As far as we can tell, the cell does not produce infectious sarcoma virus, but the aberrant particles are visible, indicating that at least some of the virus genome is present. In this figure, you can see representatives of the two RNA viruses —the sarcoma virus and leukemia virus— both of which are RNA-based. The sarcoma virus is the one which produces the transformation of ordinary tissue culture cells; the leukemia virus by itself, in an ordinary tissue culture, does not transform the cells.

II. A Mechanism of Cellular Transformation

With this introduction, I wish now to turn to the discussion of the possible way in which the RNA oncogenic Type C viruses were conceived of as introducing the necessary information into a cell to transform it from normal to neoplastic growth. The basic idea is that somewhere in the RNA of this sarcoma virus is contained a suitable collection of genes (one gene or several) which code for certain kinds of proteins, which, in turn, transform, or make, the necessary components of the cell membrane so that it no longer can receive the message to cease growing, with the result that it continues to grow and overgrows its neighbors. This last process is pretty far along in the phenotype, or read-out, of the results of the oncogene which is presumed to be present in this RNA virus. How can such a transformation in an animal cell be produced, in which the genetic information is not stored in RNA but, rather, in DNA? The suggestion was made, about six years ago, that RNA viruses, in order to reproduce themselves, had to first make a copy of their RNA into DNA, and then from that DNA, using a DNA-dependent RNA polymerase, could make more RNA, which, in turn, can create more virus (1). RNA virus replication was blocked by actinomycin D, which we know com-

plexes only with DNA and not with RNA or the enzyme, and, therefore, there must be a DNA molecule in the cycle of RNA virus replication. This was the basic idea for introducing the suggestion. About three years ago, it was shown that the RNA viruses contained in their protein structure an enzyme which was capable of copying an RNA template into a DNA strand (2, 3). This was then called RNA-instructed DNA polymerase (RDP), being given the name reverse transcriptase; for a while, it appeared that only tumor viruses and only tumor cells contained the reverse transcriptase activity; however, it has since turned out that this type of activity has been shown to reside in several different kinds of protein, from different sources. There appear to be multiple enzymes, with different template specificities, and different substrate specificities. I will use the term "reverse transcriptase activity" to cover the whole group, and refer to it only in terms of being able to copy an RNA strand into a DNA strand, without trying to specify the details of the template specificity, the primer specificity or substrate specificity.

How can this enzyme be important in the transformation process? One can imagine its importance in the viral replication process, but how can it be important in transformation? (4) Figure 4 shows, schematically,

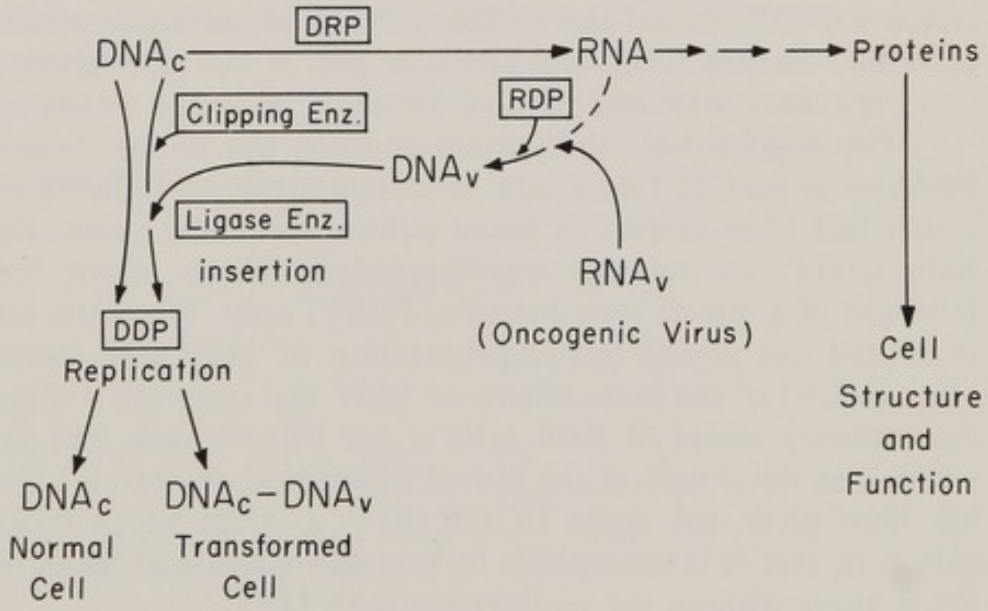


FIG. 4. Scheme for possible function of RDP in oncogenesis by RNA viruses.

a possible function of reverse transcriptase (RDP) in oncogenesis by RNA viruses. You can see herein also the cell replication process, with the DNA of the cell being copied in bits and pieces into RNA by a DNA-dependent RNA polymerase (DRP), which gives the messenger RNA, which, in turn, through multiple steps makes the necessary proteins for structure, enzyme and cell construction. This is the normal flow of

information in the replication of a cell. If, however, you infect the cell with an oncogenic RNA virus—*i.e.*, insert some RNA information, if there is such a thing as reverse transcriptase activity, then RNA could be copied back into DNA, and it could also copy the viral RNA. If the genes responsible for the lifting of the restraint on growth are contained in that viral RNA, it would be copied into the DNA by the enzyme. Then, there are at least two other enzymes involved. One of them would have to break open the DNA; this is called a *clipping enz.* in figure 4. Another would seal up the new piece of DNA from the virus into the cellular DNA; this is called a *ligase enz.* in figure 4. It so happens that enzymes of this type have been known for some time, but in the last few months I have heard descriptions of very specific enzymes from the SV40 virus, for example, and from other sources (6, 7, 8, 9). There appears to be an enzyme which clips a piece of DNA at a specific place and allows the right sets of base pairs to be exposed to complete a covalent linkage so a piece of new DNA can match up with it. This would be followed by another enzyme. There are thus at least two more enzymes involved in this insertion process to get the viral RNA information into the cellular DNA and thus make a permanent change in the cell. If the information came from an oncogenic RNA virus it would have to go through the enzyme (RDP) to get there. This is what focused our attention on that particular enzyme. You will see later that it will be necessary to expand that view somewhat and involve the other enzymes as well.

The basis for this additional concern lies in the discovery here in Berkeley a year or two ago of a variant strain of Balb/3T3 mouse cells which had been carried in tissue culture for many years. Normally this Balb strain can undergo transformation, that is, focus formation, by infection of a mouse sarcoma virus (MSV) only. When the sarcoma virus is diluted out of the mixed preparation of MSV and murine leukemia virus (MLV) to the point where no MSV and only MLV virus is present, the ordinary strain of Balb cells is not transformed. The new cell type which was developed at the Naval Biomedical Research Laboratory and has been given the name UC1-B (8) is a strain which differs from the parent in that it is susceptible to transformation and focus formation by MLV alone without the co-infection with MSV.

As will be discussed in detail later, drugs have been found which can inhibit this MLV transformation without a correspondingly large inhibition of the replication or multiplication of the leukemia virus itself. Thus, evidence, has been obtained for the participation of additional enzymes in the transformation process, other than those required for replication of the virus, which are sensitive to this class of drugs (rifamycin derivatives). It is these enzymes which have yet to be identified and to which we will refer again later.

III. RNA-Instructed DNA Polymerase (RDP) and Inhibitors

Here I will discuss primarily the nature of the RNA-instructed DNA polymerase and how we can block it from functioning. If we can prevent the RDP from functioning, the information from an RNA oncogenic virus will not be able to get into the DNA of the cell, and, therefore, the cell could not be transformed. The presence of this particular enzyme is not a sufficient condition for transformation but it is a necessary one for transformation by an RNA virus. If this function is stopped, there would then be some way of blocking the transformation of mammalian cells into tumor cells by an exogenous RNA virus.

A. Drugs

We then turned our attention to chemical methods of inhibiting that particular enzyme action (10, 11). It did not take long after the description of the RDP to have a number of materials appear which seemed to have some specific inhibitory action on the reverse transcriptase. The material which turned out to be one of the most effective was a modification of an antibiotic which had been developed for TB infections. (The rifamycin B itself was discovered about 1958 in a Mediterranean soil and was found to be particularly effective against tuberculosis). This was one member of what has turned out to be a class of antibiotics known as the ansa-antibiotics because of the nature of their structure. Four of these ansa-antibiotics, including rifamycin, are shown in figure 5. The characteristic

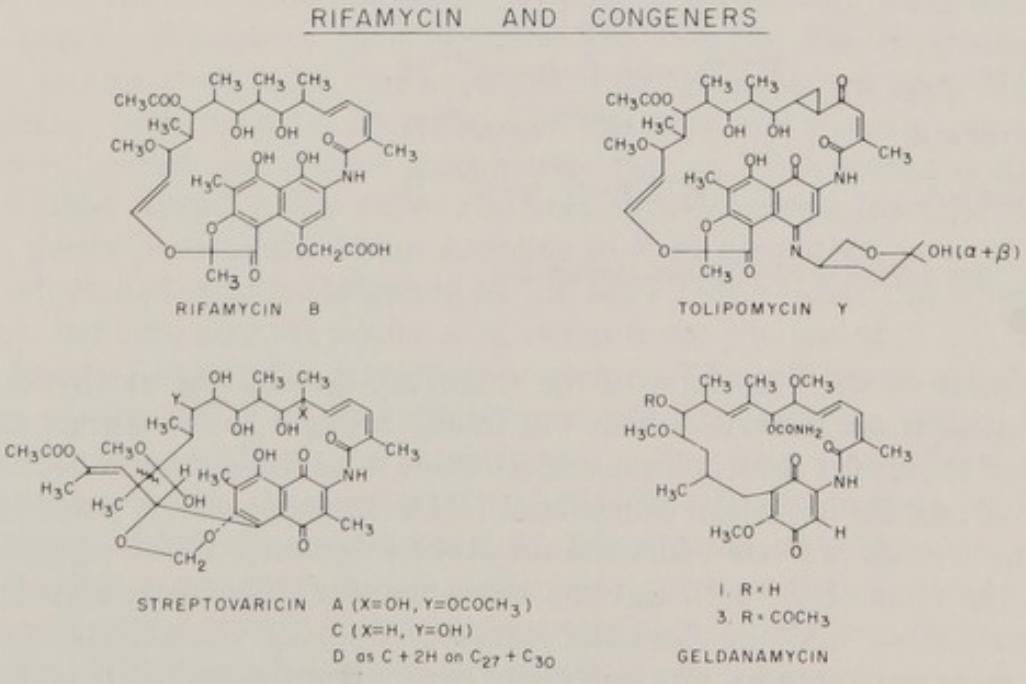


FIG. 5. Four ansa antibiotics, including rifamycin.

structure of these antibiotics is an aromatic ring which is bridged by a large aliphatic ring, which is called the ansa ring because of the nature of its bridging effect. It turned out that the material which first showed up as an inhibitor of the RDP function was a derivative of rifamycin B.

It was also shown that it was possible to make many chemical modifications of the basic rifamycin B structure, with many functional groups. It turned out that most of the derivatives were those of substitution at the No. 3 position of the naphthalene ring, where a whole series of derivatives can be made. Figure 6 shows some of the various derivatives which have been made from the rifamycin B, and most of them are actually

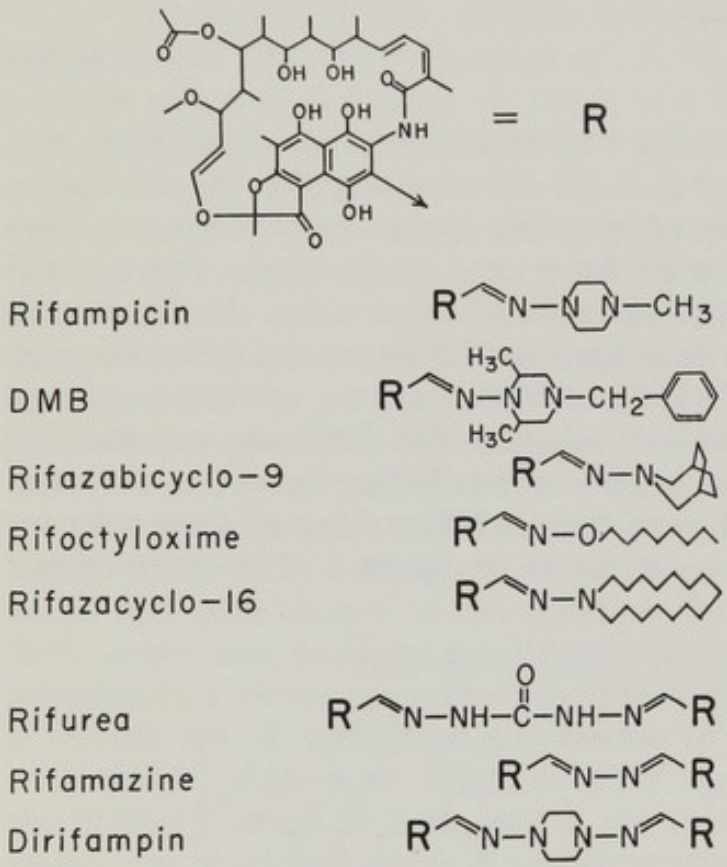


FIG. 6. Some synthetic modifications of rifamycin.

derivatives of the 3-position of the rifamycin through the aldehyde. The rifampicin is the material which was finally brought to the market as the drug of choice for tuberculosis, and its mode of action had been delineated as involving the inhibition of bacterial DNA-dependent RNA polymerase. In our studies we have focused on RNA-instructed DNA polymerase from the virus or the cell, and you might wonder what the relationship is between these and the bacterial enzyme. Both of these materials are nucleotide polymerases, and one might expect that the antibiotic inhibitors for nucleotide polymerases might have some relationship to each other

(12, 13). That is at least part of the reason that the rifampicin and its derivatives were tried on the RDP. It turned out that the dimethylbenzyl-desmethyl rifampicin (DMB) is very effective on the MSV RDP at almost as low a dose as rifampicin was effective against the DNA-dependent RNA polymerase in *Escherichia coli* and other bacteria. And it is much less effective against other mammalian polymerases.

This type of observation created the opportunity for the chemists and biochemists to synthesize additional active compounds, which, of course, are still basic modifications of the original structure of the rifamycin. With the exception of the dimethylbenzyl compound (DMB) and the rifoctyloxime compound, which we obtained originally from the manufacturer (Gruppo Lepetit, Milan, Italy) they were made in the laboratory in Berkeley (14). It turns out that I will discuss the DMB, as that is the one we received first and the one which is also non-toxic to the mammalian cell systems which we are using. The rifoctyloxime, for example, turned out to be toxic to the mammalian cell. The most important of the various derivatives which have been synthesized in Berkeley is the rifazacyclo-16 which is even more effective as an inhibitor of the reverse transcriptase enzyme activity than the DMB (14). I wish to describe more of the chemistry involved in these syntheses and the biological observations as well.

In order to study the effect of these drugs on the activity of the enzyme, it was necessary, of course, to have some purified enzyme available. The typical method by which the enzyme was first demonstrated and on which most of the work in the literature today has been based, was dependent upon a preliminary purification of the virions themselves. Then, the suspension of the virus particles is broken up, disassembled, with suitable detergents, thus liberating the enzyme. The incorporation of the various nucleotides into nucleic acid can be measured upon various templates, such as poly rA:oligo dT. The assay is actually not very definitive, except that it does demonstrate that an RNA strand is copied into a DNA strand. It was difficult to obtain clean enzyme from the virion itself, partly because the virion contains in it its own RNA as well. The cells which had been transformed by the MSV (MLV) were our principal source, but here also the presence of virion is not eliminated.

Figure 7 shows the extraction procedure. The cell layers, which are grown in the cell medium, are scraped off the bottles in which they are grown; the cell-free supernatant contains the virus. Most of our work has been done with the cell extracts. After the cells are washed, we get a cell pellet which can be broken up and precipitated with ammonium sulfate. The ammonium sulfate pellet was resuspended in a detergent (Triton X-100, for example) and the material was then centrifuged. The supernatant is called the cell extract. In effect, the result is a Triton X-100 supernatant either from the cell or from the virion. However, the cell

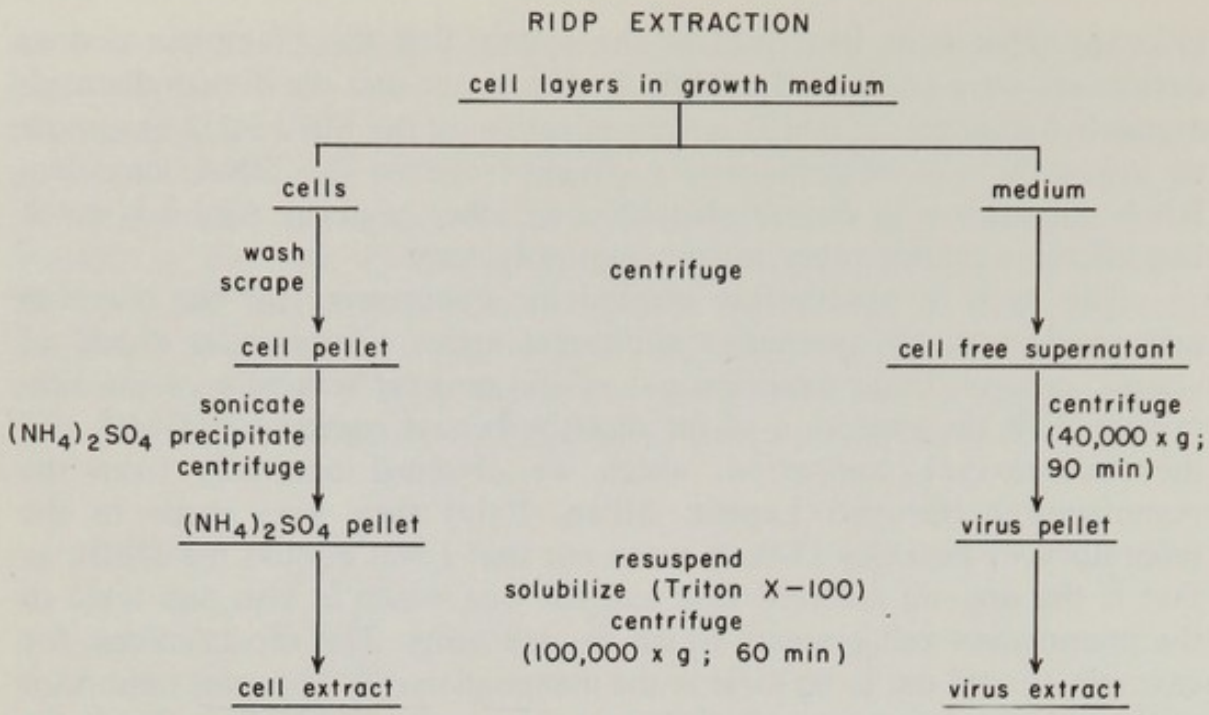


FIG. 7. Extraction scheme for RDP.

extract has a much higher total enzyme activity than the virion extract (15).

Further purifications to obtain more highly purified material have turned out to be not as simple as we had hoped. The enzyme itself is very lipophilic, which is one of the reasons for the use of detergent in the first place. It turned out, however, that the activity of the enzyme itself seems to be dependent—in fact, it is very dependent—on the presence of the detergent (16). This is not due to solubilization in detergent micelles. The detergent, of course, does help to solubilize the enzyme and take it out of the particulates so it won't centrifuge down, but it also is essential for the full activity of the enzyme itself. We learned of the effect of the detergent on enzyme activity by trying to separate out the enzyme activity from the detergent extract. When we performed that operation, we lost the enzyme activity. Then, we considered that perhaps that was merely the enzyme precipitating, but that turned out not to be the case. It was the enzyme being inactivated by the absence of a suitable lipophilic material. We were able to show this by adding detergent back again to the extract and recovering the enzyme activity.

B. Detergents

This phenomenon, showing the recovery of enzyme activity with detergent addition, is shown in figure 8. The detergents we used for this experiment were nonionic and all of them contain a polyethylene glycol chain. So, we then introduced polyethylene glycol itself as a material in the solution, and the polyethylene glycol does not activate the enzyme.

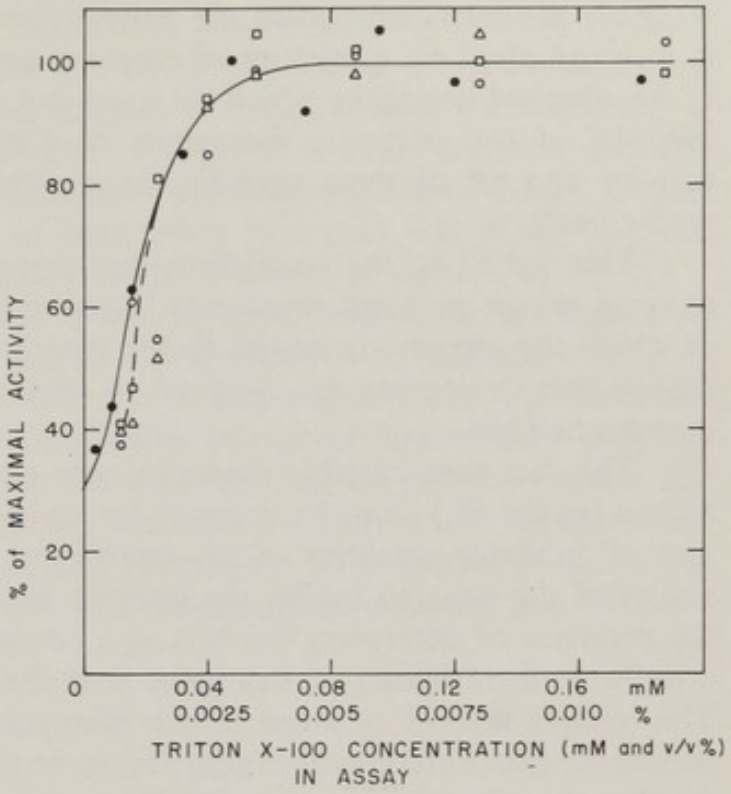
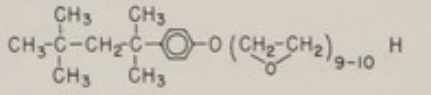


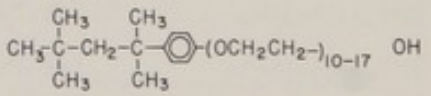
FIG. 8. Activation of RDP by nonionic detergents.

However, the detergents which contain the polyethylene glycol do activate the enzyme. The recovery of enzyme activity is approximately one-third to one-half of the original activity present. The same effect is observed no matter which nonionic detergent is used, and the activation curve of the enzyme activity is dependent upon the *molarity* of the detergent and not on the critical micelle concentration (16). The formulas of the nonionic detergents which we have used in these experiments are shown in figure 9. The first two detergents (Triton X-100 and Triton

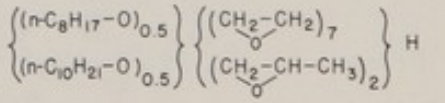
Triton X-100
(Rohm and Haas)



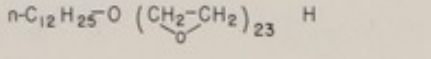
Triton X-1017



Triton DN-65
(Rohm and Haas)



Brij 35
(Sigma)



Polyethylene
Glycol-400

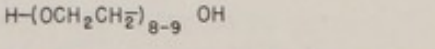
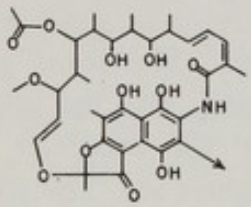


FIG. 9. Structural formula of nonionic detergents used in RDP activation. Those shown with ethylene oxide components indicate the composition of the starting monomer; those shown with polyglycol formulas indicate materials which have been purified after polymerization.

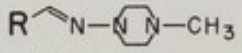
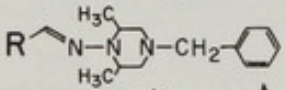
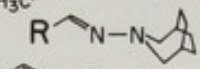
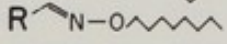
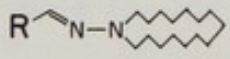
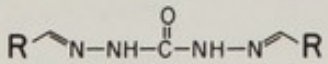
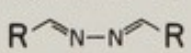
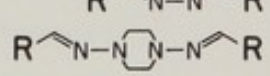
X-1017) are alkyl aromatics on polyethylene glycol; the Triton DN-65 is a mixed alkyl on a mixture of ethylene and propylene glycols. Brij-35 is the simplest detergent which we used, but it has a long ethylene glycol tail. All of the nonionic detergents do have an effect on the enzyme activity and all of them activate the enzyme to the same extent on a *molar basis.*

This led us to the conviction that there was a lipophilic site in the enzyme which perhaps represented, and might be identical with, a site at which the enzyme is bound to the membrane. This would also be the reason that detergents are required to remove the enzyme from the cell membrane (16).

The fact that variable detergent was always present in the enzyme assays (in the lit.) helps to account for some of the variability of activity and of inhibitor qualities which were reported for various drugs. The ability of the drug to inhibit the enzyme activity is also dependent upon the presence of detergent, but it is at a completely different level (critical micelle concentration) of detergent that the drug inhibition is affected. The relative activity of some of the rifamycin derivatives on the enzyme action, at two different detergent levels, is shown in figure 10. You can see that the rifoctyloxime and the rifazacyclo-16 are the most active. Even



= R

		Partition ratio	$C_{1/2}^{*}$(0.005)	$C_{1/2}^{*}$(0.025)
Rifampicin		14	>100	---
DMB		1700	22	73
Rifazabicyclo-9		>2000	12	36
Rifoctyloxime		---	6	48
Rifazacyclo-16		>2000	4	115
Rifurea		22	27	60
Rifamazine		34	21	35
Dirifampin		49	18	35

$^{*}C_{1/2}$ = the concentration of rifamycin derivative in μg/ml required to produce a 50% inhibition of RIDP activity. The numbers in parentheses are the concentrations of Triton X-100 in percent (v/v).

FIG. 10. Relative activities of rifamycin derivatives in RDP inhibition.

the large dimeric molecules are active. Notice also the amount of drug required to reduce the enzyme activity to one-half in the presence of the detergent (Triton X-100). The amount of drug required for inhibition is higher at the higher detergent level. This gave us some concern, because in the literature you will find that there are many reports of drug activities without careful specification of how much detergent was used. You can see how sensitive this inhibition is to the presence of detergent.

Why should the drug inhibition be dependent upon the detergent concentration? The obvious answer turned out to be that the detergent forms micelles and the micelles are, in effect, a lipid phase. The drug has lipid solubility and goes into the micelles in competition with the enzyme. In effect, there is a competition between the micelles and the enzyme for the lipophilic drug. This is actually what the situation is, and the inhibition titration of the RDP by DMB and rifazacyclo-16 is shown in figure 11. There were two different levels of detergent concentration and two dif-

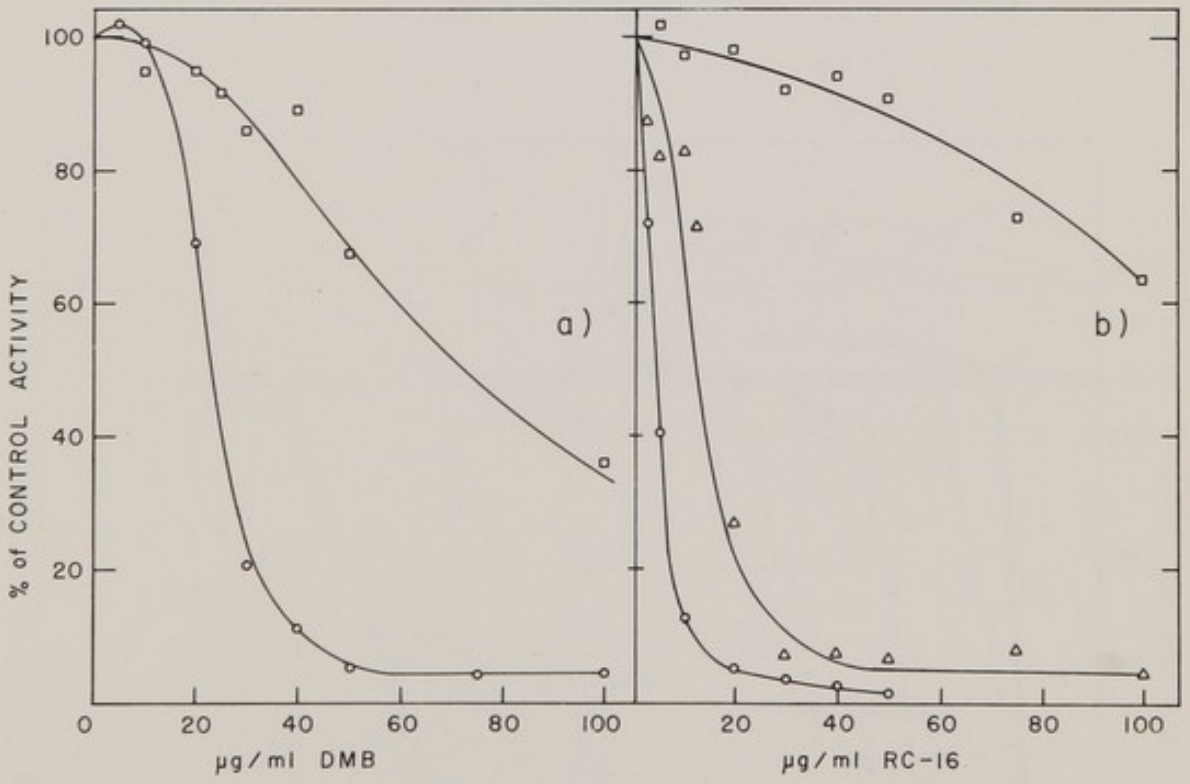


FIG. 11. Titration inhibition of RDP inhibition by DMB and RC-16 at three Triton X-100 concentrations.

ferent drugs. You can see that the rifazacyclo-16 (the circles) at very low detergent levels is extremely active. This accounts to some extent for the arguments that the chemists and biochemists have been having as to which of the drugs are effective and which ones are not. The question now

arose as to whether we could show this was micelle extraction of the drug from the enzyme. The results of this experiment are shown in figure 12. Here we have only one drug present (rifazacyclo-16) and three different detergents, and we are showing the percent of inhibition of the control activity as the function of the amount of detergent used. We have a constant amount of drug and are changing the amount of detergent. As we increase the amount of detergent, the drug becomes totally inactive, and almost 100% of the enzyme activity is exhibited; at lower detergent levels, the drug is working. The same effect shows for all of the various detergents tried – Brij-35, Triton X-100, and Triton DN-65. You can see also that the drug ceases its activity in proportion to the amount of micelle formation. I believe that there is little doubt that these two phenomena are related to each other. The drug is removed from the enzyme by extraction into the micelles. The evaluation of a drug-enzyme interaction, therefore, depends a great deal upon nature of the drug and the nature of the detergent which is in the medium and the concentration of the detergent in the medium.

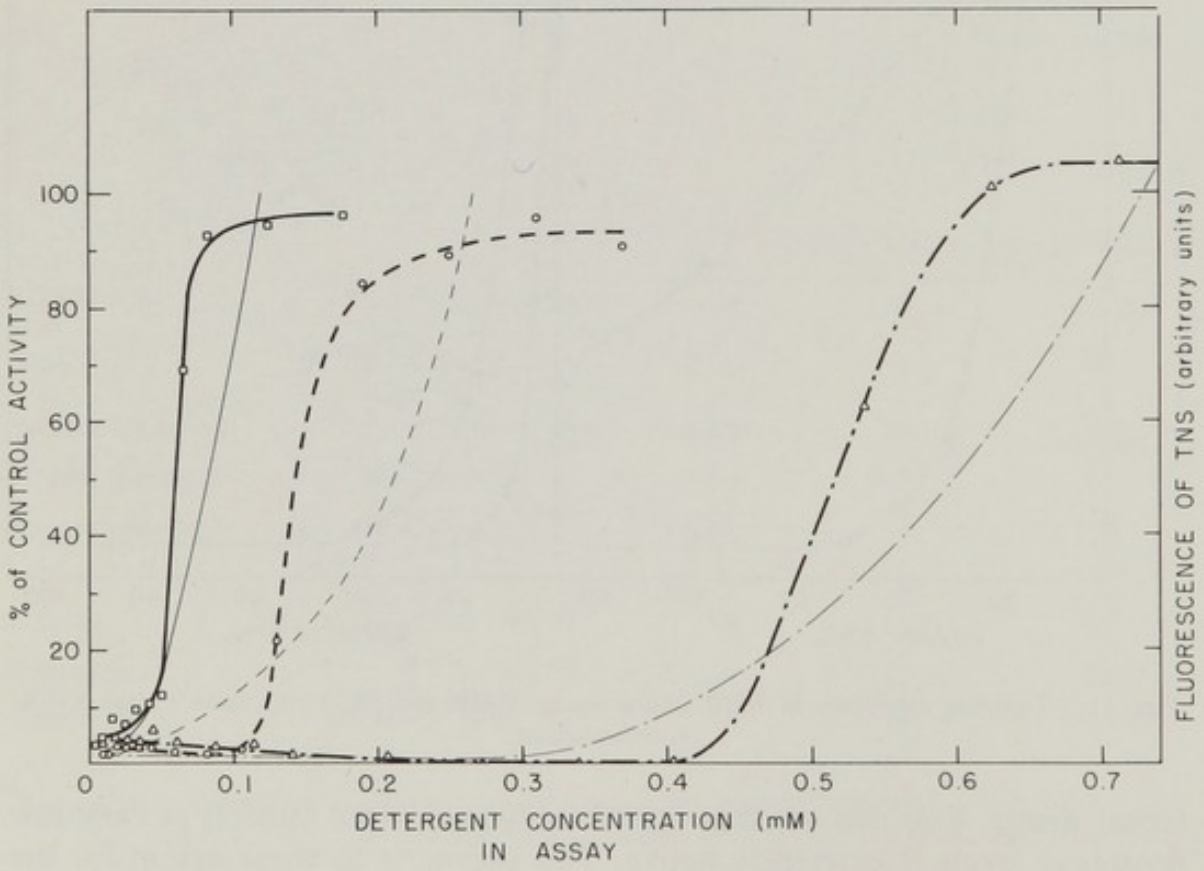


FIG. 12. RDP inhibition by RC-16 and detergent micelle formation by Brij-35, Triton X-100 and Triton DN-65 detergents.

The next step was to show that the drug actually is in the micelles. The results of an experiment in which the drug is passed through a gel permeation column in the presence of detergent is shown in figure 13. When there are no micelles, the drug comes out as free drug; as the amount of detergent is increased to where most of the detergent is in the micelles, most of the drug comes out in the detergent micelles.

We thought for some time that other components of the assay mixture might affect the drug activity, and, in fact, they do. The bovine serum albumin (BSA) has a very high lipid content, and it can act in the same way that the micelles do; the effect of solubilization of DMB with BSA is shown in figure 14. The tritiated drug (DMB), without any BSA, comes out as a simple molecule very late. As BSA is added, the drug comes out

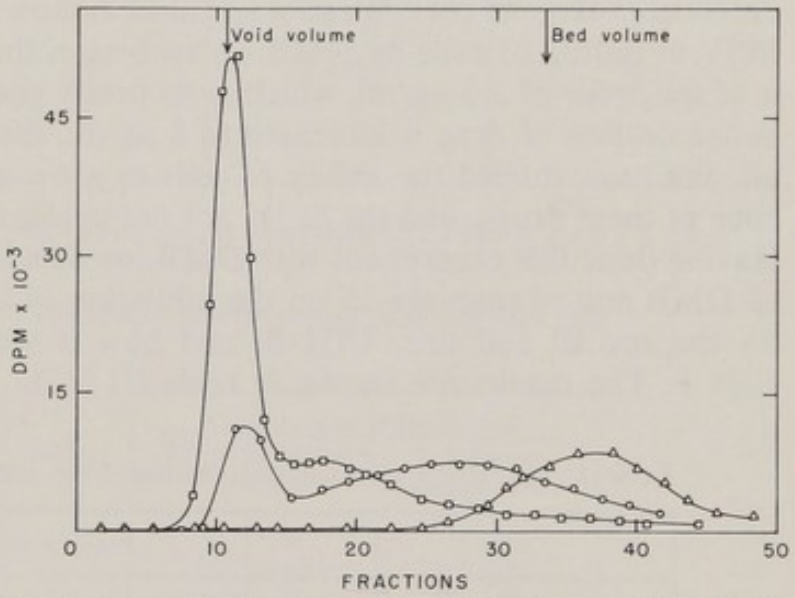


FIG. 13. Solubilization of DMB in Triton X-100 micelles by gel filtration (Sephadex G50).

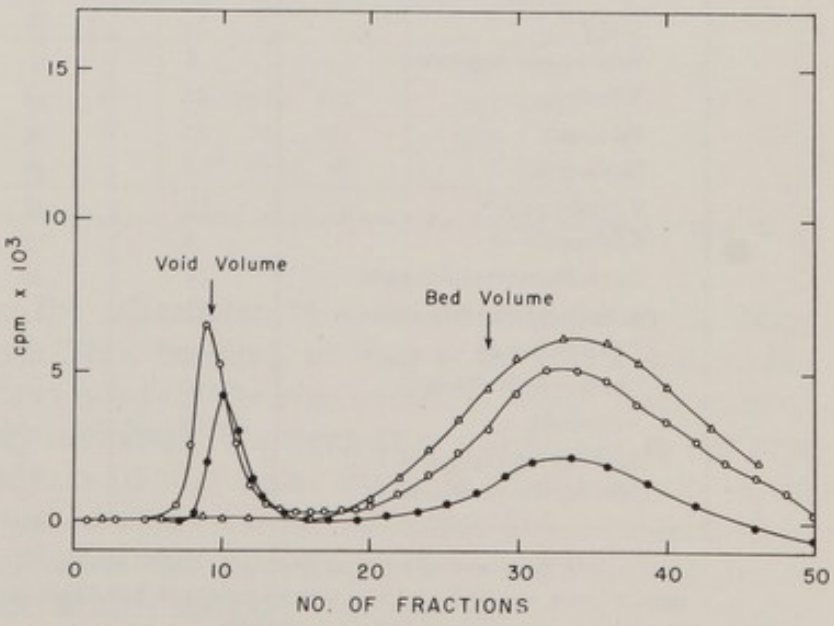


FIG. 14. Solubilization of DMB by BSA, gel filtration (Sephadex G50).

with the BSA peak and the higher BSA ratios give more of the drug in the peak. Here, again, is another variable which must be considered in these enzymatic assays.

We have shown that there are several drugs which are potent enzyme inhibitors. Is this a clue as to the ability of the drug to prevent the focus formation, *i.e.*, to prevent the transformation of a cell into a cancer cell by the virus? This would require a measurement of the ability of the virus to transform cells in the presence of different amounts of drug, without affecting the viability of the cells themselves. In other words, you mustn't hurt the ability of the cell to grow and divide, *i.e.*, relative insensitivity of DDP and DRP. Table 1 shows some of the more recent results, comparing RDP inhibition by seven different rifamycin derivatives and three different detergent concentrations. Table 2 shows the focus inhibition of MSV in Balb/3T3 cells by DMB. In each case, the level of 50% inhibition is of the order of 2-3 μg/ml, which gives pretty good control, and when the concentration of drug is increased to 8 μg/ml, there are no foci present at all. We have studied the ability of cells to grow and multiply in the presence of these drugs, and the drugs are not cytotoxic at low concentration. Having done this experiment with DMB, we decided to compare the effects of DMB and rifazacyclo-16 on the inhibition of focus formation of MLV on the special cell line, UC1-B, and how it was affected by amphotericin B. The results are shown in table III (17).

Table I. Concentration (μg/ml) yielding 50% inhibition of the RDP[1]

	Triton X-100 concentration		
Rifamycin derivative	0.005%	0.0125%	0.025%
DMB	22	25	73
Rifaldehyde octyloxime	6	16	48
Rifamazine	21	22	35
Rif-urea	27	24	60
Dirifampin	18	17	35
Rifazabicyclo-9[2]	12	12	36
Rifazacyclo-16	4	12	115
Dansyldesmethylrifampicin	11	20	85
Aminodesmethylrifampicin	55	44	135
DMB-oxidized[3]	–	100	–
Desmethylrifampicin	>100	–	–
Rifampicin	>100	400	–
Spin-labeled rifampicin 1	–	–	>275
Spin-labeled rifampicin 2	–	–	500

[1]Assays were done as described in Methods. DMSO was 1.0% or 1.0%/100 g derivative, whichever was greater. Protein was 0.45 μg-2.5 μg with an activity of 200-62 pmol/hr/g.
[2]Tetrahydrofuran was used instead of DMSO.
[3]Quinone form of DMB.

Table II. Focus inhibition of MSV in Balb/3T3 cells by DMB.

Experiment number	DMB[1] (μg/ml)	Average no. of foci	% of control foci
I	0	43	100
	5	6	14
	10	0	0
II	0	597	100
	2	301	50
	5	16	2
III	0	150	100
	3	70	47
IV	0	159	100
	6	13	8
	8	0	0

[1]Drug was present in the medium throughout the experiments, the duration of which was 7 days.

Table III. Effects of amphotericin B and rifampicin derivatives on Moloney leukemia virus transformation of UC1-B cells.

Rifampicin derivative	μg/ml	Average no. of foci formed	
		With amphotericin B (1 μg/ml)	Without amphotericin B
		%[1]	%[1]
Dimethylbenzyldesmethyl rifampicin	0	298 (100)	287 (100)
	3	180 (60)	234 (80)
	6	42 (14)	157 (54)
	12	0 (0)	0 (0)
Rifazacyclo-16	0	298 (100)	287 (100)
	3	284 (94)	291 (91)
	6	30 (10)	251 (86)
	12	0 (0)	0 (0)

[1]Figure in parenthesis: percent of control. Focus inhibition assay was done as described in table II.

We have expected the rifazacyclo-16 to be an even better inhibitor of focus formation than DMB because, as seen in table I, it will inhibit the RDP activity at about one-fifth the concentration which was required for DMB to produce the inhibition. We were surprised to note in our first experiment with rifazacyclo-16 that equal amounts of rifazacyclo-16 (in this case 6 μg/ml) produced much poorer inhibition (14% inhibition) of focus formation than DMB at the same level (46% inhibition). It was at this point that we suspected that the failure of rifazacyclo-16 to live up to its promise as indicated by its enzyme inhibiting capability when used on

whole animals might be attributed to its inability to penetrate the cell membrane. It had been previously shown that amphotericin B, a fungicide, when used on yeast in conjunction with other antibiotics (18, 19, 20) enhanced the effect of antibiotics; this was believed to be by virtue of its ability to allow the antibiotic to penetrate the cell membranes in this case. It was for this reason that amphotericin B was tried in the focus inhibition test as well.

C. Other Enzymes

We have just now begun to expand our efforts, now that we have determined a method for getting the drug into the cells. We now want to extract the enzyme to which the drug has been bound. There is a reason to believe that one of the other enzymes, other than the RDP, is even more sensitive to the drugs than is the reverse transcriptase. There are at least two other enzymes involved – one that breaks the DNA strand and the other that inserts information into the DNA. One or the other of the second stage enzymes in the insertion of new information from an RNA or DNA source is apparently even more sensitive to the drug than is the reverse transcriptase.

Reference to figure 4 clearly shows the place in which these additional enzymes play their role. We have called them, in figure 4, "clipping" enzymes, of which the restriction enzymes are a specific example. Of course, the restriction enzymes are only a specific type of a general class of nucleases called endonucleases which will clip one strand of a double strand (6). If, however, there is a specificity at the site of clipping such that both specific sites are within a limited number of bases of each other, then both strands of the double strand will be clipped in such a way as to provide sticky ends with a specific base sequence to be exposed, of anywhere from four to eight nucleotides. These specific sticky ends would then be found in both the parent DNA as well as in the DNA copy of the viral RNA if they are both clipped by the same clipping enzyme with this same specificity. This would provide a specific mechanism for inserting the viral information in the form of the DNA copy with the proper sticky ends to find a site in the cell DNA. Following this annealing would come the ligase enzyme which would then link the newly found matching base sequences by a homopolar linkage, completing the insertion process.

IV. Chemical and Viral Carcinogenesis

Our primary concern has not been viral oncogenesis but chemical carcinogenesis. At about the same time that I learned of the reverse

transcriptase I also learned that a tumor which had been generated by dimethylbenzanthracene and carried as an ascites for some time had the RDP activity in it. It was this information which really began our work on viral enzymes, which was preparatory for chemical carcinogenic research.

As this work progressed, we were pleased to learn that a relationship between chemical carcinogenesis and viral carcinogenesis has long been known. Perhaps some of the earliest work in this field was first unequivocally demonstrated by F. DURÁN-REYNALS as early as 1957 (21) in which the combined effects of chemical carcinogens and virus infection were described. Another unequivocal demonstration that the administration of both chemical carcinogen and oncogenic virus enhances the probability of appearance of neoplastic transformations appeared in 1965 (22). Much of this work has recently been reviewed by MARÍA DURÁN-REYNALS (23). Most recently, this synergism has been described on tissue cultures from a variety of cell lines (24). Here, again, not only was a synergism demonstrable but in some cases in which the virus was not added exogenously the administration of the chemical was able to produce the release of the virion into the medium (25), suggesting that the viral information was present in the cell in some form, perhaps as a plastid or even in the nuclear DNA itself (26).

A rather comprehensive review of the relationships of various endogenous viral informational fragments, particularly in chick embryo cells, has been prepared by WEISS (27). Here, again, it is evident that a variety of chemical carcinogens, including condensed ring systems, can induce the release of various forms of virions from such cells, depending upon the particular informational content of the cells. Apparently these bits of virion information are widely distributed in the fowl population, both domestic and wild, and are normally not visible in the phenotype until some special coincidence of circumstance makes their appearance possible.

Perhaps one of the most illuminating bits of tissue culture work indicating the relationship between a chemical carcinogen of the type in which we are interested, namely, the condensed ring hydrocarbons, and viral oncogenesis is some very recent work describing this synergism as a function of the order of addition of the two materials (28). Using rat embryo cells maintained for as many as 40 passages it has been shown that the chemical carcinogen 3-methylcholanthrene (MCA) at suitably chosen doses is not effective in producing transformation of this tissue culture *unless* the culture has previously been exposed and infected by a rat-adapted Rauscher leukemia virus (RLV). Furthermore, since treatment by the MCA prior to RLV infection does not produce the synergism, even after a significant number of passages with the virus, the indication is that no long-lived effect of the MCA, either in the form of an activated intermediate or in the form of a preliminary action on the cellular compo-

nents is crucial. On the other hand, since treatment with MCA following infection with this RLV will show transformation and usually not before several passages after the treatment with MCA, the suggestion here is clearly that the carcinogen acts directly on the package of oncogenic information contained in the RLV. It is for reasons such as this, but long before this particular information appeared, that our chemical carcinogenic activities were directed toward the interaction of the chemical carcinogen with a package of cancer producing information as represented by the oncogenic virus.

In the work reported here, I have another bit of evidence to add, demonstrating the relationship between viral and chemical carcinogenesis. These data are shown in figure 15. We have a strain of rats which upon two injections of dimethylbenzanthracene will within six weeks all develop mammary tumors. This is a good animal system for examining chemical carcinogenic effects. I was anxious to see if the drug would have any effect

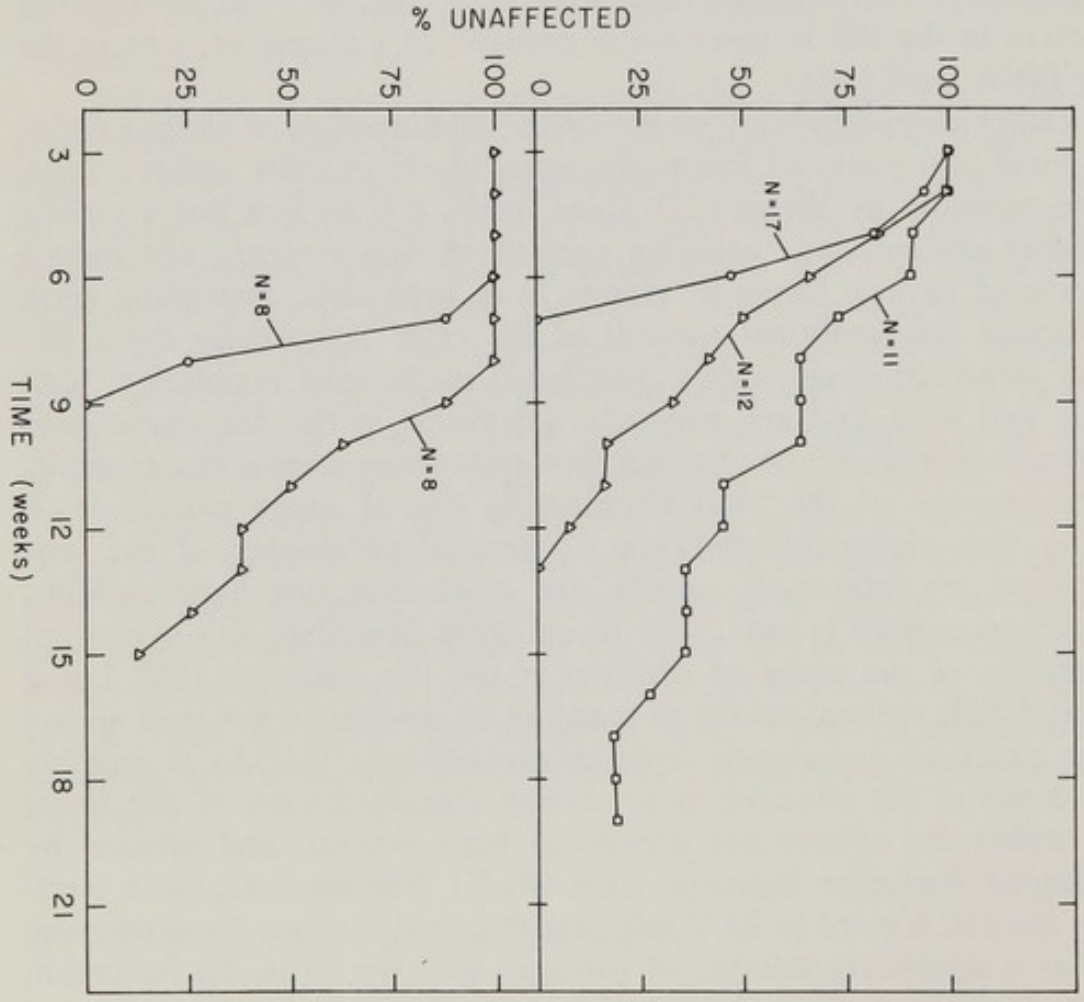


 FIG. 15. Prophylactic effect of DMB against DMBA and TMBA in rats.

in the live animals, and the results of this experiment show what happens when the drug is given to the animals after the injection of the carcinogenic material. The carcinogen is injected during the first week, and the drug is given at several different concentrations and rates for the following 10-12 weeks. The number of control animals (the animals which received no drug) are shown in the circles. The animals in the upper part of figure 15 are Sprague-Dawley rats with the carcinogen and DMB compound injected intraperitoneally; the animals in the lower part of figure 15 are Long-Evans rats injected with trimethylbenzanthracene and DMB-rifampicin injected intraperitoneally.

You will note that in both cases the onset of tumors is later with the injection of the drug and the average lifetime of the animals is from about eight weeks to eleven to thirteen weeks. The onset of the tumors is slower and the development, is slowed up (29). The drug does not cure the tumors, or stop them, but it slows them down in their onset and progress. This may be due to the fact that the chemical carcinogenesis does not involve the insertion of a new piece of RNA or DNA into the cells, but the chemical allows it to became expressed in some way.

The reason that the drug acts at all in the chemical carcinogenesis is that in order to keep the cancer growing at its full speed, the RNA-instructed DNA polymerase must play a role in the replication and division of the tumor cells. This gives us some justification for saying that the drug does indeed slow down the onset of the chemically induced tumor and slow down its ultimate development.

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CONTINUATION OF FRANCISCO' DURÁN-REYNALS STUDIES ON VIRAL ONCOGENESIS[1]

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CONTENTS

Continuation of Francisco Durán-Reynals' studies on viral oncogenesis[1]

I. Introduction

In 1945 when DURÁN-REYNALS first formulated the concept (1) that cancer may be induced by ordinary, presumably non-neoplastic viruses, he suggested that this could be the case in man and that human neoplasia may be caused by ordinary human viruses such as the influenza viruses, the herpes-viruses and viruses of the pox group like vaccinia. Twelve years later he provided the first evidence for the role in the neoplastic process of an ordinary virus widely present in man. This was his last original contribution and the last example of his resourcefulness and ingenuity in submitting theoretical concepts to the experimental test.

From the facts available, including his own work, DURÁN-REYNALS had come to the conclusion that the virus of choice, for these experiments was vaccinia. This was in the 1950's, and by then it was already evident that the poxviruses were exceptional in the variety of acute and neoplastic effects they could induce. The ability of animal poxviruses to induce both acute and neoplastic effects in the same host, and either benign or generalized progressive tumors in different hosts was well known (2). It was also known that man is the natural host of a neoplastic poxvirus which causes the human skin tumor, molluscum contagiosum (3), and that man is susceptible to two poxviruses of cattle which are indistinguishable in many respects except that one is vaccinia and the other causes the skin tumors in humans called milker's nodes (4).

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However, of all the poxviruses infective in man, vaccinia was specially qualified for the studies DURÁN-REYNALS had in mind. It was the most suitable experimentally because it probably has the widest host range. Since it was used the world over for smallpox vaccination and is highly contagious in man, vaccinia was likely to be the poxvirus most widely present in humans. Consequently, it was important to determine whether or not it was potentially neoplastic, especially in view of evidence that it might be. It was well known that the acute effects of vaccinia in human and animal skin are preceded by a proliferative response often indistinguishable from pre-malignant neoplasia, and there was also evidence that malignant tumors had been observed to develop in man at the site of the skin lesions caused by smallpox vaccination. Although in recent years a significant number of these tumors have been reported, DURÁN-REYNALS knew of only a few cases which for the most part had not been reported. However, he was certain that these tumors were highly significant in that they seemed to reveal effects of vaccinia which under the right conditions would be consistently repeated.

Demonstrating this became his main objective, and to achieve this objective he looked for a host in which vaccinia would induce the acute skin lesions it induces in humans vaccinated against smallpox. Mice pretreated with cortisone proved to be the most suitable experimental model for this purpose; and for the next step DURÁN-REYNALS took recourse to a nearly forgotten effect of chemical carcinogens.

II. The Combined Acute and Neoplastic Effects of Viruses and Chemical Carcinogens

The induction of virtually every type of tumor in a large number of species by a large number of chemical compounds is probably the subject that has been most thoroughly investigated in cancer research. However, these chemicals have certain effects which, although seemingly related to their tumor-inducing properties, have never been regarded in general as more than interesting, though somewhat marginal, observations.

These effects were first demonstrated by TEAGUE and GOODPASTURE in 1923 (5), who found that tar painting drastically enhanced the susceptibility of rabbit and guinea pig skin to the acute effects of the virus of herpes simplex; thus, whereas the virus had virtually no effect on normal skin, in tar-painted skin it induced severe, widespread herpetic lesions which were usually followed by generalization of the virus and death, apparently from encephalitis, of the host.

Fifteen years later, in 1938, ROUS and KIDD (6) reported that tar painting also increased very significantly the susceptibility of rabbit skin to the neoplastic effects of the virus of Shope papilloma; and similar

results were reported soon after (7, 8) in rabbits painted with methylcholanthrene or with benzpyrene.

In 1938 AHLSTROM and ANDREWES (9) demonstrated that chemical carcinogens also enhanced the neoplastic effects of a poxvirus; the virus was that of Shope fibroma, which, in rabbits inoculated with tar or with methlycholanthrene, induced generalized, progressive, apparently malignant tumors.

In 1952 DURÁN-REYNALS and BRYAN (10) reported that methylcholanthrene painting activated latent fowlpox virus in chickens and enhanced very significantly the neoplastic effects of this virus on the skin. the skin.

From these various studies it appeared that chemical carcinogens enhanced the acute effects of an acute human virus and the neoplastic effects of three animal neoplastic viruses, two of which belonged to the poxvirus group.

However, DURÁN-REYNALS was the first to demonstrate that an acute virus infective in man enhances the neoplastic effects of a chemical carcinogen. With these studies he also demonstrated that the development of malignant tumors at the site of vaccinia-induced skin lesions can be a consistently reproducible, predictable event.

III. The Combined Neoplastic Effects of Vaccinia Virus and 3-Methylcholanthrene (MCA)

Mice that received MCA paintings followed by cortisone and by the inoculation of vaccinia into the MCA-painted skin, developed severe virus-induced skin ulceration which healed with a large, florid, hyperplastic scar. In most mice the scar evolved into a malignant tumor in a few weeks; in the remaining mice this neoplastic change appeared to be a matter of time, since it was observed as late as 10 months after virus inoculation.

These results were reported by DURÁN-REYNALS in 1957 (11) in the last paper he gave personally before the long illness from which he died in March of 1958.

These studies were continued, and it was found that by changing the order of treatment similar, though more pronounced, results could be obtained with much less MCA.

A. Method of Treatment

In all the experiments described below the mice used were females about 3 months of age and, unless otherwise specified, were treated by

the method previously described (12).

The experimental mice (Group C-V-MCA) received: 1 mg daily for 5 days of cortisone injected subcutaneously; vaccinia inoculated into the skin of the flank the day of the last cortisone injection; and 1 painting daily for 5 days of 10% MCA in benzene over previously shaved flanks and back, beginning the day after virus inoculation. Control groups include: Group C-MCA, which received cortisone followed by heat-inactivated vaccinia and by MCA paintings; and Group MCA, which received live vaccinia or no vaccinia, the results being the same in either case.

The effects of this treatment were first observed in random-bred CFW mice (12), which were the mice originally used by DURÁN-REYNALS. Results disclosed the induction by the virus of extremely severe skin ulceration which evolved on healing into malignant tumors; the presence of the virus also enhanced the leukemogenic response. The mice thus developed a high incidence of vaccinia skin tumors and of leukemia. However, vaccinia appeared to be more effective in enhancing the leukemogenic response in the mice without tumors, of which 60% (6/10) developed this condition, whereas only 25% (6/24) of the mice with tumors also developed leukemia.

Results in mice of the various MCA-painted control groups free of vaccinia infection also revealed that these animals developed a comparatively low incidence of skin tumors, usually benign, and also a low incidence of leukemia, but that none appeared to develop both skin tumors and leukemia.

These studies also demonstrated that the presence of vaccinia in MCA-painted skin resistant to its acute effects did not influence the neoplastic response, as was demonstrated in mice that received no cortisone or in mice that received cortisone but had been previously immunized against the virus (12). Consequently, it appeared that the acute lesion induced by vaccinia in the painted skin determines its potentiating effect on the neoplastic response; and it also appeared that this was determined to a large extent by effects of the carcinogen on the development of the lesion.

B. The Effects of MCA-painting on the Response of the Skin to Vaccinia Infection

Studies with several inbred and hybrid mice (13) confirmed the results observed in CFW mice, in that all of these animals were resistant to vaccinia inoculated into the skin of the flank unless they were pretreated with cortisone. Thus, the hormone lowered this resistance to the extent of causing the virus to induce a skin lesion that involved superficial ulceration which persisted for 10-15 days. This lesion heals with rather

inconspicous scars which disappear in a few weeks or days; no further local changes are observed.

MCA-painting changes these effects of vaccinia, as it enhances very significantly their severity. In MCA-painted skin the virus induces a much more extensive lesion that usually involves deep ulceration and heals in 20-30 days with the typical large, florid, hyperplastic scar from which tumors will eventually develop.

However, these effects were not of the same order of magnitude in all the mice. Cortisone revealed in different mice different levels of skin susceptibility to vaccinia, and this determined the degree to which the carcinogen enhanced the severity of the skin lesion. Consequently, the lesions in MCA-painted skin varied in severity in different mice, revealing the various ways in which vaccinia influences the skin neoplastic response in conjunction with the carcinogen.

C. The Effects of Vaccinia Infection on the Neoplastic Response of MCA-painted Skin

The development of tumors from the vaccinia scar in MCA-painted skin involves the scar changing into single or multiple papillomas which desquamate and are eventually followed by late malignant tumors. This neoplastic change has been observed in virtually all mice in which vaccinia induces the typical ulcerative lesion in MCA-painted skin (13), but the more severe the lesion the more often this change is likely to occur and the more severe are the tumors involved (fig. 1). Therefore, if the ulceration is very severe, it is invariably followed by the neoplastic change. This change occurs so rapidly that often the scar is hardly noticeable because of the speed with which the papillomas develop; these papillomas, which are usually multiple, are soon followed by malignant tumors in most mice. If the ulceration is weak, it heals only occasionally with a permanent scar which several weeks or even months later might develop into a single, short-lived papilloma.

A study of the histological changes that determine this neoplastic response at the site of severe vaccinia lesions in MCA-painted skin (14) disclosed that these changes involve first the destruction by the virus of the epithelial elements, which is followed by regeneration and the formation by the new epithelium of keratin cysts that tend to coalesce and subsequently open into the skin surface to constitute the early papillomas. These changes were correlated with the rate of replication and the presence of vaccinia in the skin from the day of virus inoculation (14). Results revealed that viral replication was very much increased by the carcinogen and that vaccinia could be recovered from the skin for about 14 days. Since the first histological changes apparently leading to papi-

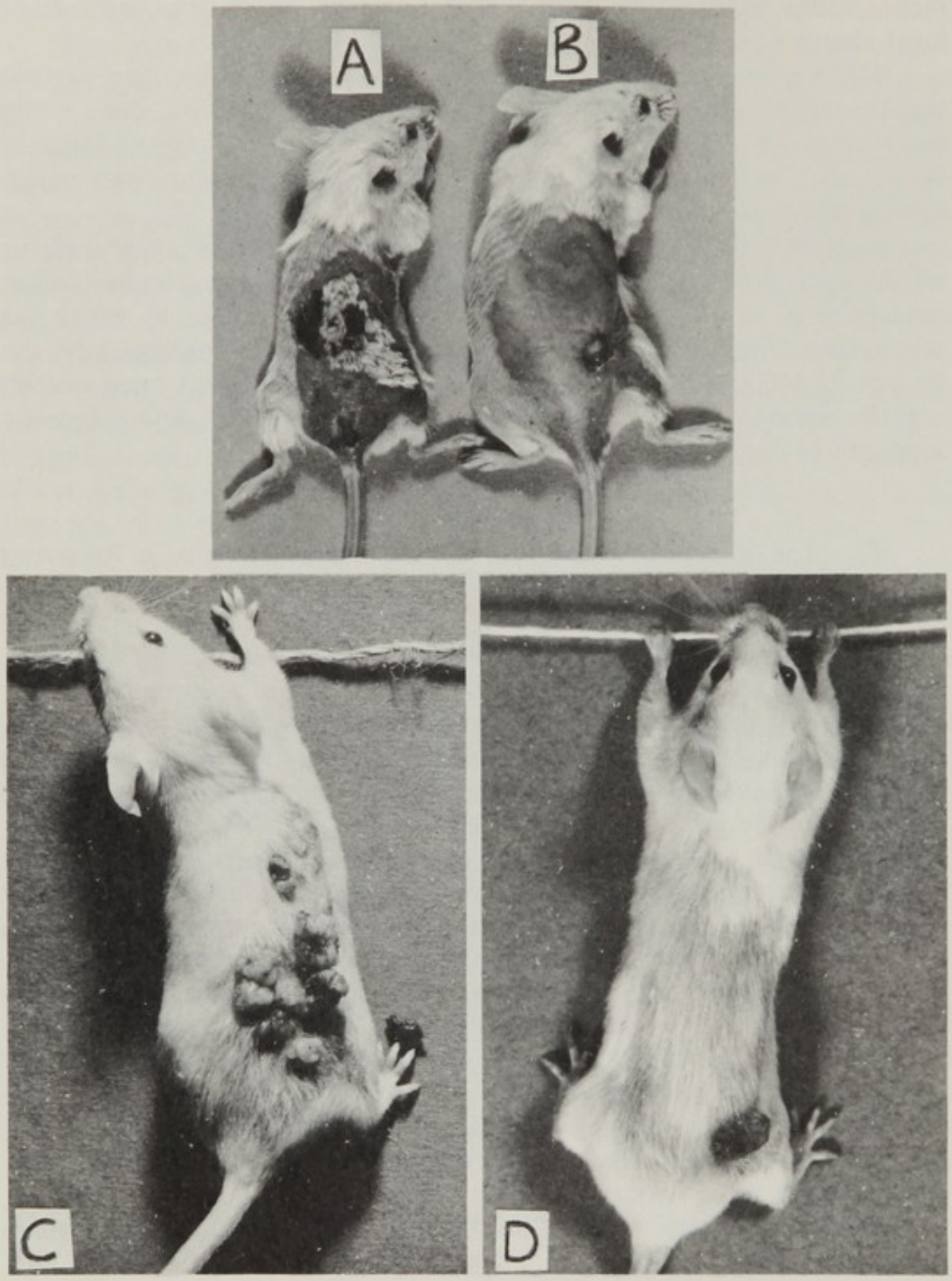

FIG. 1. *A and B.* Vaccinia-induced skin lesion in a BALB/c mouse *(a)* and in a BALB/c X AKR F_1 hybrid mouse *(b)* 15 days after virus inoculation. *(c)* Multiple tumors in the area of virus inoculation at the site of the lesion in a BALB/c mouse 7 weeks after virus inoculation. *(d)* Single tumor at the site of the lesion in an F_1 hybrid mouse 10 weeks after virus inoculation. The mice received cortisone followed by vaccinia inoculation and by MCA paintings over flanks and back (Group C-V-MCA).

lloma formation were observed to occur from the 7th to the 12th day after virus inoculation, it appeared that these changes had started while the presence of infective virus could still be demonstrated in the skin.

The neoplastic change in the vaccinia scar occurs in MCA-painted skin that is susceptible to the carcinogen, and in this respect it will be noted that the virus lesion extends only over a fraction of the painted skin. However, vaccinia infection appears to deprive the non-infected skin of its susceptibility to the carcinogen. Thus, in mice that develop a high incidence of tumors in the painted skin without as well as with vaccinia, it has been noted that without vaccinia the tumors develop at random over the painted skin and with vaccinia they develop selectively from the vaccinia scar and tend to be more severe (fig. 1).

The neoplastic change in the vaccinia scar occurs also in MCA-painted skin resistant to the carcinogen. Thus, in addition to determining the site and the severity of the tumors, vaccinia infection can also determine their actual occurrence in the painted skin.

These effects of vaccinia must now be distinguished from those of the susceptibility of the skin to the virus. The latter can be observed only in mice that received MCA paintings alone, since it appears that in these animals the skin responds to the neoplastic effects of the carcinogen only if it is susceptible to vaccinia infection.

D. Correlation Between the Skin Response to Vaccinia and MCA Painting; Inverse Correlation Between the Two Skin Responses and the Leukemogenic Response

In view of the apparent antagonism observed between skin tumors and leukemia in MCA-painted CFW mice, the experiment was first repeated with inbred AKR mice to determine whether the high susceptibility of these mice to leukemia would be associated with skin resistance to MCA painting, and whether vaccinia infection would overcome this resistance. Results revealed that AKR mice are completely resistant to skin tumorigenesis by MCA painting and that vaccinia cannot overcome this resistance because the skin is also impervious to the acute effects of the virus.

To further elucidate these findings these studies were extended to include inbred mice other than AKR (which are susceptible to spontaneous leukemia), as well as mice susceptible to leukemia induced by MCA painting and mice resistant to both.

A synopsis of results observed in these mice (13) as well as in hybrid mice (15) and in random-bred CFW mice (12) is presented in figure 2; these results concern the relative severity of the vaccinia-induced skin ulceration in Group C-V-MCA as determined by the average size of the

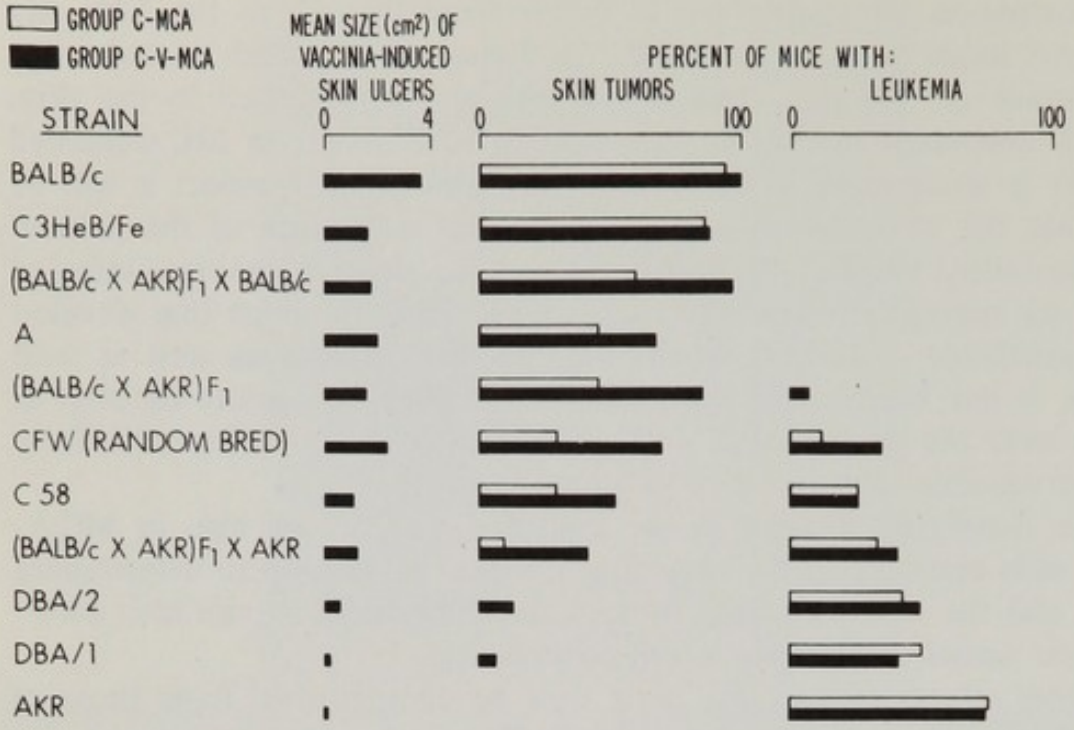

FIG. 2. Synopsis of results observed in mice of different genetic constitution: in mice of 7 inbred strains (13) that include BALB/c and AKR mice; in (BALB/c X AKR) F_1 hybrids and in mice of the F_1 X BALB/c and of the F_1 X AKR backcross generations (15); and in random-bred CFW mice (12). The mice received cortisone followed by heat-inactivated vaccinia and MCA paintings (Group C-MCA) or cortisone followed by live vaccinia and MCA paintings (Group C-V-MCA).

lesions; skin tumor incidence in control Group C-MCA and in Group C-V-MCA; and leukemia incidence also in Groups C-MCA and C-V-MCA.

The mice are listed in diminishing order of skin tumor incidence in Group C-MCA, and it will be seen that the level of this incidence corresponds to the severity of the skin response to vaccinia in Group C-V-MCA.

These results thus reveal a correlation between the susceptibility of the skin to vaccinia, as observed in Group C-V-MCA, and the susceptibility of the skin to tumorigenesis by the carcinogen in the *absence* of the virus in Group C-MCA. Therefore, both skin response to the virus in Group C-V-MCA and to the carcinogen in Group C-MCA range from extremely severe in BALB/c mice to no response in AKR mice. Because of these results the increase of tumor incidence by the vaccinia skin lesions in Group C-V-MCA became more significant as the lesions became less severe and the skin was correspondingly less susceptible to the carcinogen alone. However, vaccinia determined the site and the relative severity of the skin tumors in those mice of Group C-V-MCA, such as the BALB/c and C3H, in which it did not increase tumor incidence because of their high skin susceptibility to the carcinogen.

Results in figure 2 also reveal an inverse correlation between the skin response to vaccinia and MCA painting and the leukemogenic response. Thus, the 6 types of mice most susceptible both to the virus and to the carcinogen, from the BALB/c to the CFW, were resistant to leukemia in Group C-MCA and also in Group C-V-MCA, with the exception of the CFW mice, in which vaccinia enhanced the leukemogenic response, especially in the absence of skin tumors.

The remaining 5 types of mice, from the C58 to the AKR, all developed a significant incidence of leukemia in Group C-MCA. Of these mice, only the C58 also developed a somewhat significant incidence of skin tumors in Group C-MCA, although these tumors were usually single short-lived papillomas (13). The C58 and the mice of the F_1 X AKR backcross were the only ones in which the acute effects of vaccinia in Group C-V-MCA were sufficiently severe to enhance the skin neoplastic response. DBA/1 and DBA/2 mice developed no skin tumors in Group C-MCA and only a very few of these mice developed single papillomas at the site of the very weak lesions induced by vaccinia in Group C-V-MCA. In AKR mice these lesions were virtually absent and consequently none of these mice developed skin tumors in Group C-MCA or in Group C-V-MCA.

The average survival time of these mice (12, 13, 15) indicated that mots of those with tumors lived long enough to develop leukemia at the same age as those that developed this condition but no tumors, and that the latter in turn lived more than long enough to develop skin tumors since the tumors were always detected much earlier than the leukemias.

From these results it appeared that common factors determine the effects of the virus and of the carcinogen on the skin. Since one factor that could influence both effects is the immune response, and since it was feasible to determine the role of this response on the effects of vaccinia, experiments were performed to verify whether different levels of immunocompetence were responsible for the extreme susceptibility and the resistance, respectively, of BALB/c and AKR mice to vaccinia skin infection.

E. Role of Immunological Factors in the Skin Response to Vaccinia Infection

Results in BALB/c mice (13) demonstrated that cortisone reveals the susceptibility of the skin to vaccinia by suppressing the immune response, and that MCA painting increases the replication of the virus and the severity of its acute effects on the skin by prolonging the immunodepressive effect initiated by cortisone, thus delaying the antibody response to the virus.

Therefore, cortisone and MCA together induce a severe though transitory impairment of the immunological system that involves pronounced lymphocyte depletion of thymus, spleen and lymph nodes (13) and which causes the virus to induce severe skin lesions in BALB/c mice. However, this immunodepressive effect of cortisone and MCA painting was also observed in AKR mice, which revealed no significant skin susceptibility to vaccinia (13). From this it appeared that although immunosuppression is necessary for the skin to express its susceptibility to the virus, the level of this susceptibility is determined by local factors; and that consequently the skin of AKR mice is innately resistant to vaccinia and apparently also to the neoplastic effects of the carcinogen.

To determine whether such local factors regulate the skin response to both the virus and the carcinogen, the role of genetic determinants in the skin response to both agents was investigated in progeny of cross between the BALB/c and AKR mice.

F. Role of Genetic Factors in the Skin Response to Vaccinia and Methylcholanthrene Painting

In these studies (15), conducted in collaboration with FRANK LILLY, the inheritance of skin susceptibility to vaccinia and MCA painting was investigated in BALB/c X AKR F_1 hybrids (results were the same in AKR X BALB/c F_1 hybrids) and in mice of the F_1 X BALB/c and of the F_1 X AKR backcross generations.

Data are presented in table I on the incidence of malignant skin tumors observed (15) in these various hybrids and in the parental strains

Table I. Incidence of malignant skin tumors in mice of Group C-MCA that received cortisone, heat-inactivated vaccinia and MCA paintings; incidence of malignant skin tumors and of leukemia in mice of Group C-V-MCA that received cortisone, lise vaccinia and MCA paintings.

Strain	Number of mice		Percent of mice with:		
			Skin tumors		Leukemia
	C-MCA	C-V-MCA	C-MCA	C-V-MCA	C-V-MCA[1]
BALB/c	24	25	75	84	0
(BALB/c X AKR) F_1 X BALB/c	26	39	40	74	0
BALB/c X AKR F_1	31	43	19	65	7
(BALB/c X AKR) F_1 X AKR	50	107	0	18	36
AKR	25	30	0	0	66

[1] Leukemia incidence was about the same in Group C-MCA.

of Groups C-MCA and C-V-MCA to illustrate, by comparison, with the results in figure 2, the strong effect of vaccinia on the development of malignant skin tumors. The mice are listed in table I in the same order as in figure 2 to illustrate also that the inverse correlation between skin tumors and lukemia is especially evident in regard to malignant tumors.

G. BALB/c X AKR F_1 Hybrids

The skin susceptibility to vaccinia and MCA painting observed in the BALB/c X AKR F_1 hybrids of Group C-V-MCA demonstrates that skin susceptibility to both agents is dominant. However, the following differences were noted between the susceptible parental BALB/c strain and the hybrids (15): The hybrids were much less susceptible to the acute effects of vaccinia alone and to the neoplastic effects of MCA painting alone, and it was only when they received both the virus and the carcinogen in Group C-V-MCA that through the combined effects of both agents the virus induced rather severe skin lesions in which the incidence of skin tumors was almost as high as in the BALB/c parent (figure 2 and table I). The hybrids did not appear to inherit the high susceptibility to leukemia of the AKR parent since only 4% (9/206) of these animals in all the groups developed leukemia late in life (15).

H. (BALB/c X AKR) F_1 X BALB/c Backcross Mice

The skin response to vaccinia and MCA of mice of the F_1 X BALB/c backcross generation was, as expected, intermediate between that of the F_1 hybrids and that of the parental BALB/c strain (figure 2 and table I): no leukemia was observed in these backcross mice (15).

I. (BALB/c X AKR) F_1 X AKR Backcross Mice

Mice of the F_1 X AKR backcross generation segregated for skin susceptibility to vaccinia and MCA painting and for susceptibility to leukemia (15)

Skin susceptibility to vaccinia was observed in 78% (107/138) of these backcross mice, which indicates that susceptibility to the virus is determined in this system by two genes apparently independent of one another. Skin susceptibility to tumorigenesis by MCA painting was demonstrated only in mice with skin susceptible to vaccinia but, since only 52% (56/107) of these mice developed skin tumors, it appeared

that skin susceptibility to the carcinogens was determined by a single gene which is independent of the two genes that determine susceptibility to vaccinia.

However, it should be noted that most of these vaccinia-susceptible backcross mice were impervious to the carcinogen in the absence of vaccinia in Group C-MCA (figure 2 and table I). Consequently, tumorigenesis in MCA-painted skin was dependent on the vaccinia-induced lesions which in these animals were only of moderate to weak severity (figure 2). Therefore, it is conceivable that even minor differences in the severity of the lesions could determine the absence of skin tumors in the MCA-painted skin. In addition, the absence of such tumors also appeared to be influenced by the development of leukemia and by the histocompatibility-2 (H-2) type (15).

Of 138 mice of the F_1 X AKR backcross of Group C-V-MCA, 32% developed skin tumors, 30% leukemia and 10% both. This indicates that skin tumors and leukemia tended to be mutually exclusive since, if these two conditions had been randomly assorted with respect to one another, 17.1% of the mice should have developed both instead of only 10%.

Since the H-2 type of BALB/c mice is H-2^d and that of AKR mice is H-2^k, which is associated with susceptibility to leukemia, about half of the mice of the F_1 X AKR backcross were heterozygous for the H-2 type (H-2^d/H-2^k) and the other half homozygous (H-2^k/H-2^k). Results in these backcross mice susceptible to vaccinia revealed that the H-2 type H-2^k inhibited the development of skin tumors in Group C-V-MCA. Thus, total tumor incidence was 47% (21/45) and malignant tumor incidence 9% (4/45) in the H-2^k/H-2^k homozygotes, whereas in the H-2^d/H-2^k heterozygotes total tumor incidence was 57% (35/62) and malignant tumor incidence 26% (16/62). Results in mice of the F_1 X BALB/c backcross generation of Group MCA also revealed that total tumor incidence was 75% (18/24) in the H-2^d/H-2^d homozygotes and only 34% in the H-2^d/H-2^k heterozygotes.

These results seem to indicate that closely related or common genetic factors determine the response of the skin to vaccinia and to MCA painting. That this is the case appears to have been demonstrated by subsequent studies with skin-grafted mice.

J. The Response to Vaccinia and MCA Painting of Resistant Skin Grafted onto Hosts with Susceptible Skin

These studies (16), conducted in collaboration with MARTIN ZISBLATT and FRANK LILLY, demonstrate that the same mechanism appears to determine the skin response to both vaccinia and MCA painting at the

level of the target cell.

The system used involved skin-grafted mice in which the graft was not likely to be rejected. Thus, the skin-susceptible BALB/c X AKR F_1 hybrids were grafted either with resistant skin from the AKR parent or with the more susceptible skin of the BALB/c parent. The grafts measured about 2×3 cm and thus covered most of the back. After healing, the mice received cortisone followed by vaccinia inoculated *into the graft* and by MCA paintings over both grafted and host skin.

Results include the response of both grafted and host skin to MCA and also to vaccinia because the virus spread from the graft into the host skin.

In all of the hybrids grafted with AKR skin (30/30), the skin grafts responded weakly to vaccinia and were completely resistant to the carcinogen, whereas in the host skin the virus induced rather severe lesions and MCA painting induced a high incidence of tumors. In all control AKR mice homografted with AKR skin (10/10), both skin grafts and host skin were resistant to the virus and to the carcinogen.

In contrast to the resistance of AKR skin grafts in the hybrids, all BALB/c skin grafts (24/24) were highly susceptible in these animals to vaccinia and also developed a high incidence of tumors. Thus, in the hybrids grafted with BALB/c skin as well as in control BALB/c mice homografted with BALB/c skin, both grafted and host skin were susceptible to the acute effects of vaccinia and to the neoplastic effects of MCA painting.

IV. Discussion

From the findings described it appears that the skin response of mice to vaccinia infection and to tumorigenesis by MCA painting involves two apparently related and yet distinct phenomena: the association between the effects induced, respectively, by the virus alone and by the carcinogen alone; and the combined effects of the two agents on skin exposed to both.

The association between the effects of the virus and those of the carcinogen has been observed in all the mice tested so far and can be demonstrated by pre-treatment with cortisone, which unmasks the susceptibility of the skin to the virus, but only in mice whose skin is genetically susceptible also to the carcinogen (13). Thus, depending on their genetic constitution, cortisone-treated mice exhibit different levels of skin susceptibility to both agents, as can be seen by the results reported here in mice of 11 different genotypes, whose skin response to the virus and to the carcinogen ranged in a parallel manner from extreme susceptibility in BALB/c mice to complete resistance in AKR mice.

This correlation between the effects of the virus and those of the carcinogen appears to result from the two effects being determined by genetic factors that either interact with one another or are the same for both. This seems to be demonstrated by results summarized here of genetic studies with progeny of the cross between the BALB/c and AKR mice (15) and of studies with BALB/c X AKR F_1 hybrids, whose skin is susceptible to both agents, and were grafted either with resistant AKR skin or with the more susceptible BALB/c skin (16). The genetic studies revealed that it was not possible to separate by genetic techniques skin susceptibility to the carcinogen from susceptibility to vaccinia. The studies with the skin-grafted hybrids demonstrated that it was not possible to make resistant skin susceptible either to the virus or to the carcinogen by grafting it permanently onto a host with skin susceptible to both; consequently these results indicate that the effects of both the virus and the carcinogen on the skin are determined by genetic factors that govern both effects at the level of the target cell.

The combined effects of the virus and the carcinogen have been observed in all the mice in which vaccinia induces the typical ulcerative lesion in skin painted with MCA (13) and involve the enhancing effects of this lesion on the skin's neoplastic response. This enhancing effect causes the tumors to be usually malignant and can cause their actual occurrence in mice whose skin is otherwise resistant to the carcinogen. However, perhaps the most striking aspect of this effect of the virus is that it forces the tumors to develop at the site of the lesion (fig. 1), irrespective of whether the painted skin outside the lesion is susceptible or resistant to the carcinogen.

Since the frequency of these tumors is proportional to the severity of the lesion, the carcinogen contributes to their development through its immediate immunosuppressive action (13), which increases very significantly the severity of the acute effects of the virus on the skin. Thus, the acute response elicited by vaccinia in MCA-painted skin is characterized by deep ulceration which causes the apparently total destruction of the epithelial elements; and it is as the process of tissue repair begins that the newly formed epithelium undergoes the changes which, once started, appear to lead inexorably to the neoplastic transformation (14). One explanation that has often been given for these results is that the actively proliferating new epithelium is especially susceptible to the transforming effects of the carcinogen. However, if this is the case, it appears that such an effect of the carcinogen cannot be demonstrated in the absence of the virus, as indicated at least by the absence of tumors at the site of lesions grossly similar to those induced by vaccinia but induced instead by benzene inoculation in MCA-painted skin (12). Another explanation is that the carcinogen makes it possible through a mechanism so far unknown for the virus to exert a neoplastic

effect. This is consistent with the ability of viruses, of which poxviruses are an outstanding example, to induce acute or neoplastic effects; with findings by other authors which are specially relevant to these studies since they indicate that whereas vaccinia causes the neoplastic transformation of BALB/c embryo cells (17), AKR embryo cells are impervious to the virus (18); and with results in carcinogen-treated animals infected with neoplastic viruses in which the carcinogen enhances the neoplastic effects of virus (6-10).

Results presented here indicate that the H-2 type H-2^k inhibits tumorigenesis in MCA-painted skin. This was observed in mice of the (BALB/c X AKR) F_1 X AKR backcross generation and also in those of the F_1 X BALB/c backcross generation. These mice are presumably infected with the leukemogenic Gross virus present in the parental AKR strain and, since the H-2 type H-2^k is associated with susceptibility to leukemia, it is conceivable that the inhibiting effect of this H-2 type on skin tumorigenesis by MCA painting, for which there is no explanation at present, is in some way related to the association observed between skin resistance to the carcinogen and the development of leukemia.

This association has been observed in random-bred CFW mice and in mice of the F_1 X AKR backcross generation. However, the relation between skin resistance to the carcinogen and the development of leukemia has been most clearly demonstrated in inbred mice, and it will be noted that this has been further confirmed by studies in progress. It is also worth noting that the only inbred mice that do not develop leukemia and in which resistant skin has been demonstrated so far, are mice such as the B10.D2 (13) that respond to MCA painting with severe, wide-spread ulceration of the skin which tends to persist indefinitely. Whether this ulcerative response precludes the development of tumors, or whether the mice are resistant to skin tumorigenesis by MCA painting, has not been determined.

Results in inbred mice (13) summarized here indicate that MCA painting induces few or no skin tumors in mice in which it induces a high incidence of leukemia, such as the DBA/1 and DBA/2, or in mice that spontaneously develop a high incidence of this disease such as the C58, and, above all, the AKR, which represent the extreme in regard to both susceptibility to leukemia and skin resistance to the carcinogen. Since in all of these mice the skin is as best only weakly susceptible to vaccinia, it is conceivable that the development of leukemia is actually associated with resistance to the virus and that this in turn causes the skin to be resistant also to the carcinogen. Therefore, one interpretation of these results is that latent leukemogenic viruses interfere with the acute effects of vaccinia on the skin under conditions that may involve, on the one hand, a low level of skin susceptibility to both agents and, on the other, the induction by the latent virus of a high incidence of leukemia which

may be an indication of the virus being abundantly present in the host. This would explain the association of resistant skin with spontaneous leukemia, which is virus-induced, and also with leukemia caused by the carcinogen, since it appears that in mice, chemically-induced leukemia (19) like radiation-induced leukemia (20, 21), involves the activation of latent leukemogenic viruses.

In apparent contrast to these findings but perhaps related to them are results indicating that vaccinia infection enhances the leukemogenic response. This has been observed by MAZURENKO (22) and later by ILIE (23) in mice inoculated neonatally with vaccinia and has also been observed in this laboratory (12) in MCA-painted CFW mice (figure 2). The mice in all of these studies rarely developed spontaneous leukemia, and in CFW mice MCA painting induced a low incidence (5-8%) of this disease (12). CFW mice also differ from the other mice tested so far in that their skin susceptibility to the carcinogen alone is rather low by comparison with their skin susceptibility to vaccinia, which is very high (figure 2). Thus, it may be speculated that the absence of leukemia contributes to the virus inducing a severe lesion in MCA-painted skin, and that, in a host weakly susceptible to the carcinogen, this lesion enhances the skin's neoplastic response or the leukemogenic response. This is what seems to happen in MCA-painted CFW mice in which the vaccinia lesion was much more effective in enhancing the leukemogenic response in the absence than in the presence of skin tumors (12).

The implications of these results in regard to the effects of vaccinia in man are an open question. Clinical observations duplicate some of these results. Thus, both malignant tumors at the site of vaccination and the development of leukemia following vaccination have been reported. The tumors are of different types and it is interesting that they can develop in more than one vaccination site. This has been observed in three patients, one of whom developed two tumors (28), another two tumors (35) and the third three tumors (35) at the site of successive vaccinations. Thus, 62 tumors have been reported in 58 patients (24-41). Forty-eight of these patients have been reported in the United States and of these 35 were reported in 3 papers (27, 30, 35) by authors who were specially interested in collecting these cases; and this suggests that a systematic search would reveal that such cases are more common than had been suspected. The development of leukemia soon or immediately after vaccination has been reported in 18 patients (42-50); in 2 there was a previous history of chronic lymphocytosis that apparently developed into rapidly progressing leukemia on vaccination (42-50); but in the other 16 cases there was no evidence of such conditions previous to vaccination. In most of these patients the effects of vaccination appeared to be rather severe and often involved generalized vaccinia. Observations have also been reported indicating that vaccination is often followed by conditions that although

apparently benign resemble Hodgkins disease or lymphosarcoma (51, 52). Although from the information available no conclusions can be reached as to the meaning of these clinical findings, they nevertheless emphasize the significance of the experimental results presented here.

In practical terms these results strongly indicate that acute viral infection increases the cancer risk in individuals exposed to carcinogenic agents, and that consequently such exposure should be avoided, especially during the acute phase of the infection.

Theoretically, these results emphasize the complexities attending the neoplastic change as indicated by the associations and correlations, on the one hand, and the apparent antagonisms, on the other, between the various effects observed.

At any rate, these results derive from the original observations of DURÁN-REYNALS and as such they are another example of the influence his work and his concepts have and will probably continue to have for a long time in cancer research.

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THE STATE OF THE VIRAL GENOME IN SV40-INDUCED CANCER CELLS[1]

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CONTENTS

The state of the viral genome in SV40-induced cancer cells[1]

I. Introduction

Probably the best characterized tumor cells resulting from transformation events mediated by DNA-containing oncogenic viruses are those induced by the papovaviruses, simian virus 40 (SV40) and polyoma. SV40-transformed cells are usually virus-free, but there is strong evidence that the transforming viral genome persists in an integrated state in the transformed cells. It is possible, under specified conditions, to recover small amounts of infectious virus from most SV40-transformed cell lines, indicating that the complete viral genome is present but is repressed in some as yet unidentified way.

A schematic representation of an SV40-transformed cell is shown in figure 1. The transforming genome is integrated into the cellular chromosome in the nucleus at one or more sites. SV40-specific messenger RNA (mRNA) is found in both the nucleus and cytoplasm. Tumor antigen is synthesized and accumulates in the nucleus. Some type of virus-specific repressor may be present, but the evidence is not clear on this point. A multitude of changes occur at the cell surface, including the appearance of transplantation antigen, surface antigen, agglutination sites, embryonic antigens, and normal cell antigens. The cell may be immune to superinfection and, most importantly, may be malignant. By comparison, all these changes are absent or masked in a normal cell.

The experimental observations which generated the above concepts regarding the state of the transforming viral genome are considered in this paper, and directions of current research in this area are discussed.

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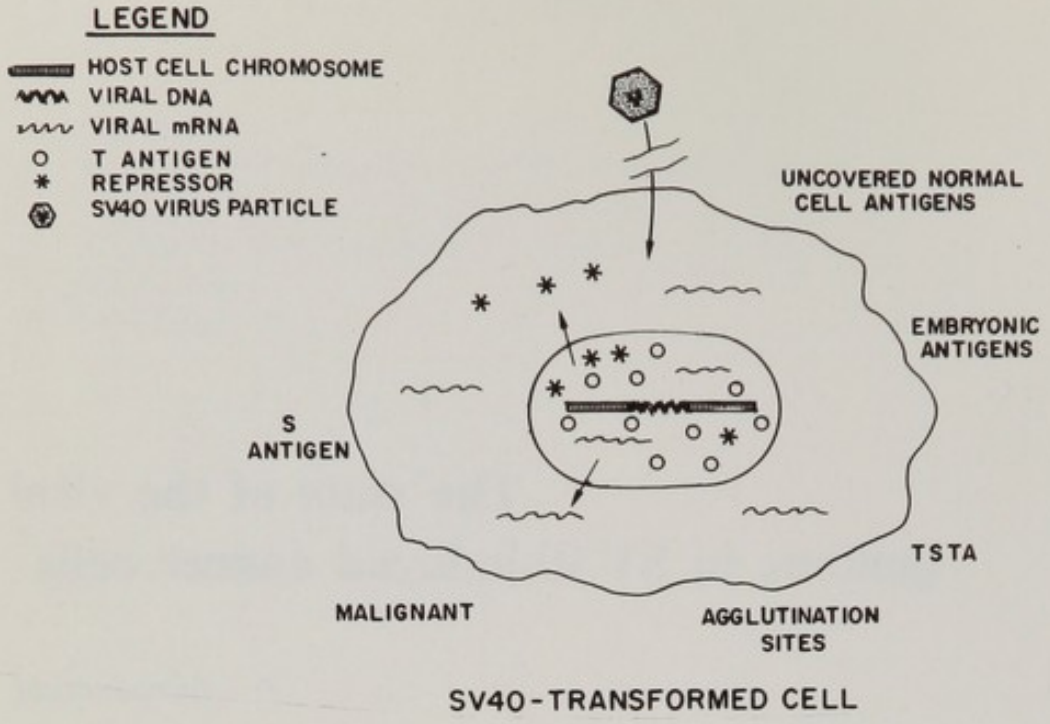

FIG. 1. Diagrammatic representation of an SV40-transformed cell.

II. Evidence for Integration of the Viral Genome

SV40-transformed cells produce infectious virus only very rarely, if at all. This fact led to attempts to determine the state of the viral genome in such cells, particularly since assays of nucleic acid isolated from SV40-transformed cells failed to reveal the presence of any free, infectious viral DNA (SABIN and KOCH, 1963b; KIT *et al.*, 1968; BOYD and BUTEL, 1972).

REICH *et al.* (1966) were the first to report that SV40-transformed hamster cells contained DNA with homology to SV40 mRNA prepared *in vitro*. WESTPHAL and DULBECCO (1968) confirmed these observations using RNA prepared with highly purified component I virus DNA as template and estimated the number of viral DNA equivalents per transformed cell as ranging from 7-58. More recent studies have resulted in estimations of only 1-9 SV40 DNA equivalents per transformed cell (LEVINE *et al.*, 1970; GELB *et al.*, 1971). Such estimates, of course, do not indicate whether or not some of the "equivalents" might be defective (incomplete) viral genomes.

The next step was to determine the state of the multiple copies of viral nucleic acid within the transformed cells. Using the DNA-RNA hybridization technique and an SV40-transformed line of mouse 3T3 cells, DULBECCO and his colleagues (SAMBROOK *et al.*, 1968; WESTPHAL and DULBECCO, 1968) established the following points about the physical state of the viral DNA: (1) It is in the nucleus; no hybridizable DNA was

detected in cytoplasmic extracts. (2) It is associated with the cellular chromosomes. Chromosomes were isolated from metaphase cells and the DNA isolated from the chromosomes was shown to hybridize to the same extent as DNA from interphase cells. (3) It is not in the form of circular molecules. Following centrifugation to equilibrium in cesium chloride in the presence of ethidium bromide, the hybridizable material was found in the cellular DNA band in the gradient. (4) It is not present in the form of free molecules the size of a single SV40 genome. The HIRT (1967) salt precipitation method, which separates large and small molecules of DNA, showed that the hybridizable regions were found in the precipitate of cellular DNA. (5) The viral and cellular DNAs are not separated by alkaline denaturation and centrifugation in a sucrose gradient. Therefore, the main conclusion to be drawn from these studies is that the SV40 DNA is covalently bound to the chromosomal DNA of the transformed cell. The sites or sites of insertion of the multiple copies of the genome is still unknown. Identical findings were obtained for polyoma DNA in similar experiments carried out in parallel with polyoma-transformed 3T3 cells (WESTPHAL and DULBECCO, 1968).

The use of interferon has also suggested the integration of the viral genome into that of the host cell. The induction of tumor (T) antigen by SV40 in 3T3 cells is sensitive to inhibition by interferon, but serial passage of SV40-transformed 3T3 cells in the presence of interferon had no effect on the synthesis of T antigen (OXMAN *et al.*, 1967). Previous investigations had established that fixation of the transformed state in the infected cell required only one cell generation (TODARO and GREEN, 1966) and that treatment of the cells as the time of infection with interferon would prevent transformation (TODARO and BARON, 1965). The use of synchronized cell cultures revealed that if the cells were not synthesizing DNA, transformation remained interferon-sensitive, but if the cells were rapidly synthesizing cellular DNA (S phase), transformation readily became interferon-resistant (TODARO and GREEN, 1967). Since there is evidence that interferon blocks translation of viral mRNA (JOKLIK and MERIGAN, 1966; MARCUS and SALB, 1966), these observations can be explained by the assumption that the viral DNA becomes integrated into the cellular chromosomes during the S phase of cell growth. Once integrated, the viral mRNA is masked by attached regions of cell mRNA so that interferon no longer recognizes it as being viral in origin.

Numerous studies have attempted to determine the degree to which transcription of viral genes occurs in transformed cells. BENJAMIN (1966) first reported that a small fraction of pulse-labeled RNA from polyoma or SV40-transformed cells was able to hybridize with the DNA of the corresponding virus. ALONI *et al.* (1968) found with the SV40 system that mRNA formed during lytic infection prior to viral DNA synthesis ("early" RNA) was different from the mRNA present after the onset of

viral DNA replication ("late" RNA). Approximately one-third of the SV40 genome was represented in the early RNA whereas at least 75% of the genome was represented in late RNA. Competition experiments between virus-specific RNA from transformed cells and late RNA from infected cells suggested that only about one-third of the genome was transcribed in the transformed cells. ODA and DULBECCO (1968) and SAUER and KIDWAI (1968) both confirmed the existence of early and late mRNA after infection with SV40. Interestingly, approximately 80% of the SV40 genome appeared to be transcribed in one transformed green monkey kidney cell line. In all cases, it appeared that the lack of expression of certain viral genes in transformed cells was at the level of transcription.

A more recent study of the regulation of SV40 gene activity in transformed cells utilized a series of SV40-transformed mouse cell lines (MARTIN and AXELROD, 1969). The extent of transcription in the individual lines varied, ranging from 30% to 100% of that seen during lytic infection. This study emphasized that the extent of transcription of the SV40 genome is variable from one transformed cell line to the next, even within a single species of host cell. SAUER (1971) demonstrated that in an SV40-transformed green monkey kidney cell line, production of late viral mRNA sequences was not prevented when DNA synthesis was inhibited. This was in contrast to a productive infection by SV40 in which inhibitors of DNA synthesis prevented the appearance of late mRNA. The mechanism(s) responsible for the apparent differences in transcriptional control is not known at this time.

High molecular weight heterogenous RNA that contains viral-specific RNA has been detected in the nucleus of transformed mouse cells (LINDBERG and DARNELL, 1970). Polysomal mRNA of lower molecular weight also contained viral-specific RNA. The authors suggested that the large nuclear molecules may be precursors of the cytoplasmic mRNA. Of particular interest was the fact that the largest molecules containing SV40 sequences were longer than one SV40 genome. These molecules were subsequently shown to carry both viral and cellular base sequences (WALL and DARNELL, 1971). Polycistronic "viral-cell" hybrid RNA molecules have also been detected in adenovirus-2-transformed rat embryo cells and adenovirus-7-induced hamster tumor cells (TSUEI *et al.*, 1972). The presence of cellular mRNA regions adjacent to viral-specific sequences is very formidable evidence in support of the concept of integration of the viral genome into that of the host cell.

Recent results from studies on the transcription of SV40 DNA during productive infection complicate interpretations of regulatory mechanisms at the transcriptional level. It appears that there is a strand-switch during *in vivo* transcription. Early RNA, synthesized prior to viral DNA synthesis, is transcribed off one DNA strand. After DNA replication has

started, "late" message is transcribed off the other strand (KHOURY and MARTIN, 1972; LINDSTROM and DULBECCO, 1972; SAMBROOK *et al.*, 1972). *Escherichia coli* DNA polymerase copies only the "early", or minus strand, and that in its entirety. In mouse cells abortively infected with SV40, the extent and pattern of transcription was almost identical to that seen in permissive cells late in infection (KHOURY *et al.*, 1972).

Transformed cells contain primarily early sequences copied from the minus strand. Using separated strands of ^{32}P-labeled SV40 DNA, OZANNE *et al.* (1972) made the interesting observation that although not more than 30% of the sequences of the early strand appear in stable species of RNA in productively infected cells, the percentages range from 40-80% in transformed mouse cells. They interpreted this as evidence that some of the viral sequences in transformed cells are "anti-late" and presumably non-informational. Most lines of transformed cells also contained RNA complementary to a small (<20%) segment of the late strand of SV40 DNA. Such "anti-late" sequences detected by OZANNE *et al.* (1972) might be the explanation for the apparently nearly complete pattern of transcription (when compared to lytically-infected cells) described for some of the transformed cell lines studied by MARTIN and AXELROD (1969).

Controls at the transcriptional and post-transcriptional levels during productive and abortive infections by SV40 and their relevance to transcription of viral information in transformed cells are currently unresolved, but are the subject of intense investigation.

III. Rescue of the Transforming Viral Genome

Cells transformed either *in vivo* or *in vitro* by SV40 are generally virus-free. However, occasional tumors and cell lines spontaneously release small amounts of infectious virus (GERBER and KIRSCHSTEIN, 1962; ASHKENAZI and MELNICK, 1963; BLACK and ROWE, 1963; SABIN and KOCH, 1963a, 1963b). Minute amounts of virus found in extracts of such tumors amounted at most to $10^{3.3}$ TCD_{50}/g of tumor tissue. Induction attempts using procedures known to induce lysogenic bacteria to produce infectious bacteriophages, such as exposure to mitomycin C, proflavine, hydrogen peroxide, and X-irradiation, were only minimally effective when applied to SV40 tumor cells (SABIN and KOCH, 1963b; GERBER, 1964; ROTHSCHILD and BLACK, 1970). Combinations of inducing agents were not more effective at induction of infectious SV40 than were the individual agents alone. In addition, there was no dose-response relationship between the concentration of inducer employed and the yield of virus obtained (ROTHSCHILD and BLACK, 1970). Yields of SV40 following induction of mitomycin C were in the range of 10-1,000

PFU/10^6 cells. Thus, only the very rare tumor cell had been induced to release infectious virus in those experiments.

GERBER (1966) discovered that recovery of infectious SV40 was more efficient if the tumor cells were placed in direct contact with susceptible indicator cells, such as primary African green monkey kidney cells. The sensitivity of the indicator cell system was increased by the use of inactivated Sendai virus to form heterokaryons (HARRIS and WATKINS, 1965) of tumor and indicator cells (GERBER, 1966; KOPROWSKI *et al.*, 1967; WATKINS and DULBECCO, 1967). The fusion technique increased the yield of virus about 1,000-fold over simple co-cultivation, but the vast majority of the heterokaryons were non-productive (KOPROWSKI *et al.*, 1967; WATKINS and DULBECCO, 1967). Although one of the most sensitive means of virus rescue currently available, it succeeds in inducing only a rare tumor cell to produce virus. The frequency of successful virus recoveries and the amount of virus rescued in the fusion experiments has been observed (1) to vary widely between clonal lines of transformed cells derived from the same parental cell line (WATKINS and DULBECCO, 1967), (2) to vary at different passage levels of the same clone (WATKINS and DULBECCO, 1967), (3) not to correlate with the number of viral DNA equivalents estimated to be present in each transformed cell (WESTPHAL and DULBECCO, 1968), and (4) not to be dependent upon the multiplicity of infection at the time transformation occurred (KIT and BROWN, 1969).

Recently, it has been demonstrated that it is possible to rescue infectious SV40 by the inoculation of chromosomal DNA from an SV40-transformed cell line into permissive simian cells in the presence of diethylaminoethyl (DEAE)-dextran (BOYD and BUTEL, 1972) following the procedure outlined schematically in figure 2. The actual DNA extrac-

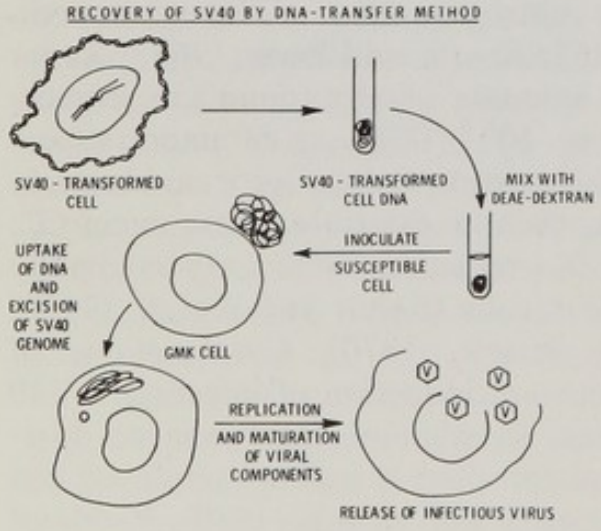

 FIG. 2. Schematic diagram of the DNA-transfer method of virus rescue.

tion procedure employed did not seem to matter, but relatively large amounts of the transformed cell DNA were required (>10 μg/culture of 10^6 cells) to effect rescue of SV40 by passage in monkey cells. Representative results are presented in table I. Virus was recovered from three different species of SV40-transformed cells by the "DNA-transfer" method; the efficiency of virus recovery varied from about 50% to 80% of the trials using various cell lines as the source of the donor DNA. It was noteworthy that the DNA-transfer method succeeded in rescuing virus from a number of cell lines which were non-yielders in fusion experiments.

Attempts were then made to ascertain the state of the infectious DNA in the transformed cell. Results are summarized in table II. It appeared that the DNA-transfer method was recovering the integrated viral genome because (1) the infectious DNA from transformed cells was found in the Hirt pellet of precipitated cellular DNA rather than

Table I. Recovery of virus from simian cells inoculated with DNA from SV40-transformed cells.

Source of cellular DNA	Species of origin	Transforming agent	Yield SV40 by fusion	No. positive experiments/ No. trials	Yield of virus (PFU*/10^6 cells) first GMK cell passage of cellular DNA extracted by:†		
					method 1	method 2	method 3
TSV-5	hamster	SV40	+	9/11	1.3×10^4††	4.5×10^3	3.0×10^3
$RIBS_{11}$	hamster	SV40	+	10/13	6.0×10^3	3.3×10^3	3.0×10^3
SRHH	hamster	SV40	0	12/15	4.6×10^4	5.5×10^3	6.0×10^4
H-50	hamster	SV40	0	5/10	9.0×10^2	2.4×10^3	1.1×10^3
SV-3T3	mouse	SV40	+	4/5	8.5×10^2	ND**	ND
VLM	mouse	SV40	0	6/10	1.0×10^3	1.1×10^3	1.1×10^3
BSC-1-S	monkey	SV40	0	2/4	8.0×10^2	1.1×10^3	ND
T-22	monkey	SV40 (T fraction, irradiated)	0	0/3	$<10^1$	$<10^1$	ND
DMBA	hamster	dimethylbenzanthracene	ND	0/4	$<10^1$	$<10^1$	$<10^1$
Ad 31 ts	hamster	Adenovirus 31 ts	ND	0/4	$<10^1$	$<10^1$	$<10^1$
SHL-7 (39)	hamster	PARA (39nT)-adeno 7	ND	0/4	$<10^1$	$<10^1$	$<10^1$
GMK	monkey	—	ND	0/6	$<10^1$	$<10^1$	$<10^1$
SV40-infected GMK (Hirt supernatant fluid)	—	—	—	6/6	4.1×10^6	3.5×10^7	3.0×10^6

*PFU= Plaque-forming units.

**ND= Not done.

† See BOYD and BUTEL (1972) for description of extraction methods.

†† With each type of cellular DNA, no virus was recovered if the DNA were treated with deoxyribonuclease prior to passage or if the DEAE-dextran were omitted at the time the DNA was applied to the simian cells.

Table II. State of the infectious DNA in SV40-transformed cells.

Sample tested	Sample should contain the following materials if present in the transformed or infected cells	Recovery of infectious virus
Transformed cells[1]		
Cell lysate	Complete virus	0
Hirt[2] supernate	Free SV40 DNA	0
Hirt pellet	Cellular DNA	+
Marmur[3]-extracted DNA	Total DNA	+
Marmur-extracted DNA, 100 °C for 15 min	Denatured linear DNA, intact circular SV40 DNA	0
SV40-infected GMK cells		
Hirt supernate	Free SV40 DNA	+
Hirt pellet	Cellular DNA, trapped SV40 DNA	0
Hirt supernate mixed with Hirt pellet from control GMK cells, 100 °C for 15 min	Denatured linear DNA, intact circular SV40 DNA	+

[1] Identical results obtained with 3 different transformed hamster cell lines.
[2] Salt precipitation method of HIRT (1967).
[3] Method described by MARMUR (1961).

the supernate where free SV40 DNA appears, and (2) the infectivity was inactivated by boiling which does not destroy free SV40 circular DNA. This technique, therefore, provided additional evidence that the resident viral genome is associated with the cellular DNA. The applicability of the DNA-transfer method to other transformed cell systems remains to be determined, although a similar approach has recently recovered infectious Rous sarcoma virus from virus-free, transformed cells (HILL and HILLOVA, 1972; MONTAGNIER and VIGIER, 1972).

"Partial" induction of one SV40-transformed cell line has been achieved by heat shock. Exposure to 45 °C for 30 min induced the synthesis of SV40 viral antigen, but not infectious virus, in transformed BSC-1 cells (MARGALITH *et al.*, 1970). The mechanism of this heat induction phenomenon is not clear, but a plausible explanation is that only a portion of the transforming viral genome has been derepressed and possibly only one of the viral coat protein polypeptides synthesized. Recent results are compatible with this hypothesis (MARGALITH *et al.*, 1972). It is not yet known whether the heat induction procedure results in the detachment of an integrated viral genome prior to capsid antigen synthesis.

IV. Mechanism of Maintenance of Integration

An important question crucial to an understanding of the process of viral carcinogenesis is the mechanism by which transformed cells

prevent the completion of the virus replicative cycle. Viral DNA is present, mRNA is transcribed, early antigens are synthesized, but no complete virions are produced. Four hypotheses can be proposed: (1) A virus-specific "repressor" is synthesized and the presence of this material blocks the replicative cycle. CASSINGENA and colleagues (1968, 1969) reported on the presence of a specific "repressor", protein in nature, in cells of different species transformed by SV40, as well as in cells productively or abortively infected by SV40. However, other investigators using a variety of cell lines and experimental approaches have failed to obtain results compatible with the existence of a cytoplasmic virus-specific repressor (JENSEN and KOPROWSKI, 1969; SWETLY *et al.*, 1969; BARBANTI-BRODANO *et al.*, 1970; BUTEL *et al.*, 1971; KIT *et al.*, 1971). (2) The transformed cells lack some essential factor required by the virus for replication. Several of the above studies showed that the majority of transformed monkey and human cell lines were susceptible to superinfection by SV40 DNA even though some were resistant to challenge with complete SV40. Therefore, it appears that transformed cells can possess all the necessary SV40 replication factors. (3) Defective viral genomes which are inherently unable to replicate because of a lack of certain genetic information are the transforming agents. Some SV40-transformed monkey kidney cell lines, as well as many SV40-transformed human cell lines, will yield infectious virus under appropriate rescue conditions (ASHKENAZI and MELNICK, 1963; KOPROWSKI *et al.*, 1967; KIT *et al.*, 1970a; BOYD and BUTEL, 1972). Therefore, one cannot conclude that permissive cells can be transformed only by defective particles. (4) An excision system required to release the viral genome from integration is absent. This possibility was investigated as part of a study designed to determine the mechanism of rescue of SV40 by the DNA-transfer method described above (BUTEL and BOYD, 1973). After inoculation of the transformed cell DNA into permissive cells, small molecular weight infectious DNA appeared in Hirt supernate fractions within 6 h, even in the presence of a DNA inhibitor (table III). Using a different approach employing tritium-labeled transformed cell DNA and nitrocellulose column chromatography, the conversion of labeled DNA in permissive monkey cells to a circular form was demonstrated. The circular DNA eluted from the column was infectious, yielding complete SV40 on passage in monkey cells. These results suggest that the integrated SV40 DNA is excised from the transformed cell DNA in simian cells and resumes a circular configuration. Similar experiments were then carried out using a single transformed cell DNA preparation and a series of SV40-transformed cell lines as recipient host cells. Preliminary results suggest that most of the transformed cell lines lack a comparable "excision system" (BUTEL and BOYD, 1973). It would be premature at this point, however, to speculate on whether the absence of the "excision system"

Table III. Conversion of infectious transformed cell DNA from large to small molecule in simian cells.

Source of cellular DNA inoculum	Donor cells		Passage in monkey cells	
	Species of origin	SV40-transformed	ara-C* present	infectious DNA in Hirt supernate, 6 h post-inoculation (PFU†/10^6 cells)
TSV-5	hamster	+	0	2.0×10^2
TSV-5	hamster	+	+	1.0×10^2
SRHH	hamster	+	0	4.0×10^1
SRHH	hamster	+	+	5.0×10^1
SV-3T3	mouse	+	+	5.0×10^1
CV-1	monkey	0	0	$<10^1$
CV-1	monkey	0	+	$<10^1$
Control SV40 DNA	–	–	0	7.0×10^2
Control SV40 DNA	–	–	+	8.0×10^2

*10 μg/ml of arabinofuranosylcytosine (ara-C) was added to the culture medium at the time of inoculation of transformed cell DNA.
† PFU=Plaque-forming units.

is correlated in any way with the maintenance of integration of the viral genome in those cells.

V. Conclusions

The evidence is strong, then, that in SV40-transformed cells, the viral genome is integrated into the chromosomal DNA of the host cell. Furthermore, it is probable that the complete viral genome is present in most transformed cell lines, although the absolute number of copies of that genome present per cell is still the subject of some controversy.

One of the most intriguing of the currently unresolved general areas pertinent to SV40-transformed cells is the mechanism by which the integration of the viral genome is maintained. Several different and potentially very informative approaches are being brought to bear on this question, including the use of defective hybrid viruses (Lewis and Rowe, 1971; Rapp, 1973), temperature-sensitive virus mutants (Kit *et al.*, 1970b; Tegtmeyer and Ozer, 1971; Robb and Martin, 1972; Butel, unpublished data) and bacterial restriction enzymes (Danna and Nathans, 1971; Morrow and Berg, 1972; Mulder and Delius, 1972). The resolution of this problem, in addition to providing insight into the process of viral-induced transformation, will also broaden our understanding of regulatory mechanisms in mammalian cells.

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INHERITED ONCORNAVIRUS GENES AND THEIR ACTIVATION

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CONTENTS

Inherited oncornavirus genes and their activation

The presence of tumour-virus genes in apparently uninfected cells was implicit in DURÁN-REYNAL'S work and ideas (1953, 1956) on chemical and viral carcinogenesis. ANDREWS (1939) had speculated on the role of latent virus infections in cancer and it was postulated by DARLINGTON (1948) that such viruses could arise from cellular genetic elements, which he named "proviruses". In recent years, studies on latent oncornavirus genomes have led to new speculation on their role in cancer in the form of the oncogene hypothesis (BENTVELZEN *et al.*, 1968; HUEBNER and TODARO, 1969) and the protovirus hypothesis (TEMIN, 1971). In this paper I shall briefly recount the evidence for inherited viral genomes in mice and chickens and how the genomes are controlled by endogenous and exogenous factors.

OBERLING and GUÉRIN (1950) claimed that chemically-induced tumours in the fowl gave rise to filterable sarcomagenic agents. This remarkable idea aroused little attention until GROSS (1958) suggested a similar phenomenon in murine leukaemias induced by X-rays. In the following year, LIEBERMAN and KAPLAN (1959) confirmed the induction of a leukaemia virus by X-irradiation. Since that time many reports have appeared on the induction of tumour viruses in apparently uninfected animals by chemical carcinogens or ionising radiations (IRINO *et al.*, 1963; TOTH, 1963; HARAN-GHERA and PELED, 1967; KAPLAN, 1967; IGEL *et al.*, 1969; TIMMERMANS *et al.*, 1969; BALL and MCCARTER, 1971). The appearance of the tumour viruses could be a result of the immunosuppressive action of the carcinogens, which would permit latent infections to become virulent, but recent reports on the spontaneous or induced activation of tumour viruses endogenous in normal embryonic fibroblast

cultures (Aaronson *et al.*, 1969; Rowe *et al.*, 1971; Weiss *et al.*, 1971) suggest, rather, that these viruses arise from genetic elements present in each cell much in the same manner as lysogenic bacteria become activated to produce bacteriophage.

I. What Is the Evidence for Oncornavirus Genomes in Normal Cells?

A. Virus-like DNA in Normal Cells

In 1964, Temin postulated the provirus hypothesis, that the RNA viral genome forms a DNA copy as a replicative intermediate in infected cells. Therefore he looked for viral DNA in infected cells using the technique of nucleic acid hybridization (Temin 1964). His results were not convincing, but other laboratories (Harel *et al.*, 1966; Wilson and Bauer, 1967) investigated the problem and showed not only that virus-like DNA was present in infected cells but that it was present in uninfected cells too. The significance of this finding was not immediately apparent but we now realise, using more sophisticated hybridization techniques, that several DNA genome equivalents of oncornaviruses exist in normal cells (Gelb *et al.*, 1971; Rosenthal *et al.*, 1971; Baluda, 1972; Varmus *et al.*, 1972a, b) so viral DNA is apparently integrated with the host cell genome.

B. Viral Antigens in Normal Cells

Dougherty and Di Stefano (1966) observed that the group-specific *(gs)* antigen (Sarma *et al.*, 1964) for avian tumour viruses was present in apparently uninfected chicken embryos. Payne and Chubb (1968) studied the inbred chicken Reaseheath C-and I-lines. Whereas the C-line was negative for *gs* antigens, the I-line was consistently positive. Moreover, crosses between the lines showed that the *gs* antigens were genetically determined by an autosomal locus with a dominant allele for antigen expression. In addition to *gs* antigens, viral envelope antigens have been found in embryonic chick fibroblasts (Weiss, Friis and Vogt, unpublished observations). *Gs* antigens have also been described in embryonic mice and hamsters (Huebner *et al.*, 1970; Taylor *et al.*, 1971), and animals which synthesize *gs* antigens early in life tend to be immunologically tolerant to those antigens. It might be thought that the *gs* antigens of the virus are coded by cellular genes, and that it is no more surprising to find *gs* antigens in uninfected cells than other cellular proteins. But there is evidence that the *gs* antigens of the virus are coded by the viral genome because they appear in mammalian hosts following infection with

avian tumour viruses (SARMA *et al.*, 1964; BUBENIK and BAUER, 1967), so the presence of indistinguishable antigens in normal cells suggests that viral genes are present in those cells. However, complete virus is not commonly produced, suggesting either that the viral genome is defective and cannot make complete virus, or that only some of the viral genes are expressed in cells.

C. Complementation of Defective Rous Sarcoma Virus

The Bryan strain of Rous sarcoma virus is defective in the sense that while it can transform cells to a neoplastic state, it cannot replicate infectious progeny (HANAFUSA *et al.*, 1963). The defect appears to be the inability to synthesize the envelope glycoprotein (SCHEELE and HANAFUSA, 1971) which is necessary for penetration of the host cell and determines the host range of the virus. Infectious stocks of Bryan strain Rous sarcoma virus (RSV) carry non-transforming "helper" viruses called "Rous associated viruses" (RAV's) which donate their own envelope components to the RSV by phenotypic mixing (HANAFUSA, 1965; VOGT, 1965). Different strains of avian oncornaviruses have envelope glycoproteins which can be distinguished from each other antigenically or by their host range and interference patterns, and they have been classified into subgroups on this basis (VOGT, 1970). The antigenicity and host-range of Byran RSV is determined by its RAV. Whenever Byran RSV is cloned free of RAV, it is unable to replicate infectious particles. For a time it was thought that Bryan RSV could produce infectious virus in the absence of helper virus (VOGT, 1967; WEISS, 1967; HANAFUSA and HANAFUSA, 1968). This virus, named RSV(O), had an unusual host-range; it was infectious for Japanese quails, pheasants and turkeys but was not infectious for most strains of chicken. Later it was shown that the infectious RSV produced in the apparent absence of helper virus was only released from certain kinds of chicken cells (WEISS, 1969; H. HANAFUSA *et al.*, 1970) containing an agent called "chick helper factor" *(chf)* which conferred the infectivitiy of a unique host range to the RSV. *Chf* was found in *gs* positive chick cells but was seldom found in *gs* negative chick cells (WEISS, 1969; WEISS and PAYNE, 1971), indicating that the autosomal gene which controlled *gs* antigen apparently controlled the presence of *chf* too. In some strains of fowl, there was a differential expression of *gs* antigens and *chf* (T. HANAFUSA, *et al.*, 1972; WEISS, FRIIS and VOGT, unpublished observations). Thus, two viral markers were found to be present in normal chick embryonic cells, the *gs* antigens, and the *chf* which provided specific envelope components for defective RSV. *Gs* positive cells from diverse breeds of chicken (WEISS, 1972) provide *chf* for detective RSV which posses identical host-range and antigenicity.

Not all defective mutants of RVA are complemented by cells expressing chick helper factor. For instance, mutants of reverse transcriptase are not complemented by *chf* positive cells (HANAFUSA and HANAFUSA, 1971; WEISS, 1973), nor are functions for cell transformation complemented by *chf*. Mutants of RSV are proving useful in the analysis of *chf* functions. Helper factors for mouse sarcoma virus have not been described.

D. Rescue of Endogenous Viral Genes by Exogenous Viral Infection

The infectious form of Byran RSV release from chf^+ cells could be indefinitely propagated in infectious form in chf^- cells provided that the cells were infected with high titres (WEISS, 1969; H. HANAFUSA *et al.*, 1970). In contrast, on solitary infection of *gs* negative cells with RSV no infectious progeny were produced. These observations indicated that the helper factor genome could be rescued and transmitted to cells lacking the factor. T. HANAFUSA *et al.* (1970) found that other strains of RSV and strains of RAV could also rescue the *chf* genome as a complete virus, called RAV-60, which was isolated and distinguished from the exogenous virus by its unique host-range. They thought that the rescue of *chf* in the form of RAV-60 might result from recombination with the rescuing virus. Recently, WEISS *et al.* (1973) have shown that genetic recombination takes place between non-defective strains of Rous sarcoma virus and the endogenous viral genome. Recombinants were isolated which carry the transforming genes of RSV and the host-range gene of *chf*.

E. Immunity to Exogenous Infection

Following the lysogenic bacteriophage model, DAAMS *et al.* (1968) have postulated that an endogenous mammary tumour-virus genome produces a repressor which confers immunity to superinfection. The susceptibility of chick cells to infection with the different antigenic subgroups of avian oncornaviruses depends on the presence of specific receptor sites at the cell surface. The receptors are genetically determined by single autosomal loci with dominant alleles for susceptibility (see VOGT, 1970; PYNE *et al.*, 1973). In contrast to other subgroups, susceptibility to the endogenous virus (subgroup E) is controlled by two independently segregating autosomal genes (PAYNE *et al.*, 1971). One gene codes for receptor sites and is dominant for susceptibility, while the other has an epistatic inhibitory effect on the expression of susceptibility. The epistatic gene is inhibitory for subgroup E only. Evidence is accumulating (CRITTENDEN *et al.* 1973; WEISS, FRIIS and VOGT, unpublished observations) that the inhibitor gene may be another expression of the *gs*

gene. It is possible that viral envelope products produced in the cell by the endogenous genome block the receptor sites to exogenous infection in much the same way that leukosis viruses interfere with superinfection by RSV (STECK and RUBIN, 1966; VOGT and ISHIZAKI, 1966). This would explain why most chicken cells are resistant to infection from without by their own endogenous virus.

F. Spontaneous Release of Complete Virus

Most inbred strains of mice which have a high incidence of leukaemia, produce murine leukaemia virus (MLV) in their tissues. This virus is "vertically" transmitted from one generation to the next. The vertical transmission may occur by *congenital infection,* but in some strains, e.g., AKR, C58, it appears to be *genetically transmitted* in the germ cells without passing through an infectious stage (ROWE *et al.,* 1971). During the life of the offspring there is a high probability that the virus will become active and spread throughout the tissues of the body. Long-term cultures of fibroblasts may also spontaneously begin to release MLV (AARONSON *et al.,* 1969; ROWE *et al.,* 1971). A similar situation pertains for mouse mammary tumour virus (HAGEMAN *et al.,* 1972); some strains are transmitted by congenital infection, either through the milk or across the placenta but other strains are transmitted genetically through the germ cells (BENTVELZEN and DAAMS, 1969). In chickens, VOGT and FRIIS (1971) found that one line which was susceptible to infection with subgroup E leukosis virus, produced about 20% embryos which were spontaneously releasing a subgroup E virus which they named RAV-0. This virus was distinguished from common congenital infections (RUBIN *et al.,* 1961) by its envelope specificity and because viraemic hens did not consistently produce viraemic embryos. In a related strain of chickens, 50% of the offspring become viraemic embryos. In a related strain of chickens, 50% of the offspring become viraemic during embryonic development and remain so for the rest of their lives (CRITTENDEN, SMITH and WEISS, unpublished observations). However, the incidence of leukosis is not notably high in this strain.

G. Induction of Leukaemia Viruses in Fibroblasts Cultures

It has been demostrated unequivocally that murine and avian embryonic fibroblast cultures can be induced to release C-type oncornaviruses after treatment with chemical or physical carcinogens and mutagens (ROWE *et al.,* 1971; WEISS *et al.,* 1971). Even chick cells which are negative for *gs* antigen and *chf* may be induced to release complete

virus. Induction of complete virus is a rare event ($1:10^5$ or less), but virus-specific products such as *gs* antigen and *chf* may be induced in a large proportion of the cells following treatment (Weiss *et al.*, 1971). Certain established mouse cell lines are much more readily inducible than primary embryonic fibroblast cultures (Lowy *et al.*, 1971; Aaronson *et al.*, 1971). In chick cells a variety of mutagenic agents was found to be effective for virus induction but in mouse cells, by far the most effective agents are the thymidine analogues, 5-bromodeoxyuridine and 5-iododeoxyuridine. Leukaemia virus induction may be readily assayed in "non-producer" lines carrying defective murine sarcoma virus genomes, by treating them with inducing agents, whereupon infectious MSV pseudotypes are released (Klement *et al.*, 1971; Aaronson, 1971). Teich *et al.* (1972) have shown that in order to activate endogenous virus, the thymidine analogues must be incorporated into the host cell DNA; the incidence of virus induction is enhanced by subsequent treatment of the cells with UV or blue light, causing breaks in the substituted DNA. Thus oncornavirus induction resembles bacteriophage induction and excision of the viral genome may be necessary for replication. However, it should be borne in mind that the induced genomes are, of course, RNA, so that transcription products only are required to produce complete virus.

II. Genetic Regulation of Endogenous Viruses

Two classes of "host" gene affect the expression of endogenous viruses.

A. Genes for Viral Activation

In some inbred strains of mice, e.g., AKR, BALB/c, leukaemia virus is highly inducible; in others, e.g., C57BL, it is not. Breeding experiments (Stephenson and Aaronson, 1972a, b; Rowe, 1972; Rowe and Hartley, 1972) have shown that inducibility depends on alleles present at two or possibly three independent chromosomal loci. There is circumstantial evidence (Rowe and Hartley, 1972) that at least one of these loci is located in the endogenous viral genome. These loci have dominant alleles for high inducibility. Similar loci may prove to control spontaneous activation of RAV-0 in chickens (Crittenden, Smith and Weiss, unpublished observations). Only a few strains of chickens produce RAV-0 and experiments are in progress to determine its genetic control.

In chickens the *gs* locus (Payne and Chubb, 1968; Weiss and Payne, 1971) controls partial expression of the viral genome. When the gs^+ allele is present, *gs* antigens and envelope antigens are synthesized

but there is no release of complete virus. The viral genome is also present in gs^- embryos since they contain equivalent numbers of copies of viral DNA (ROSENTHAL *et al.*, 1971; VARMUS *et al.*, 1972) and can be induced to produce complete virus (WEISS *et al.*, 1971). Therefore, the *gs* locus has a regulatory function. Viral gene expression appears to be controlled at the transcriptional level because virus-like RNA is not found in gs^- cells but is present in gs^+ cells (LEON *et al.*, 1972; BISHOP *et al.*, 1973; HAYWARD and HANAFUSA, 1973). It is not known whether the *gs* locus resides in the endogenous viral genome or at another locus. It is noteworthy that the *gs* alleles control expression of endogenous virus only, for exogenous viruses replicate equally well in gs^+ and gs^- cells.

Murine mammary tumour viruses may also be controlled at the transcriptional level (SPIEGELMAN *et al.*, 1972; VARMUS *et al.*, 1972b), but BISHOP *et al* (1973) have found virus-specific RNA sequences even in strains such as C57BL which do not normally synthesize viral gene products. BENTVELZEN *et al.*, (1972) believe there is just enough transcription to code for a repressor of complete replication.

B. Genes which Control the Spread of Activated Virus

Even when there is a relatively high rate of spontaneous or induced activation of leukaemia virus, the virus will not spread through the organism or the culture unless the cells are susceptible to exogenous infection. In the mouse, susceptibility is governed by the Fv-1 locus (PINCUS *et al.*, 1971) which confers resistance to N- or B-tropic viruses. In contrast to susceptibility in chickens, resistance to infection is dominant and reciprocal, e.g., an N-type cell is susceptible to N-tropic virus and is resistant to B-tropic virus. If the endogenous virus activated in an N-type cell is itself N-tropic, the virus can spread from cell to cell as an infectious agent and cause viraemia (ROWE, 1972; STEPHENSON and AARONSON 1972a). If, however, the endogenous virus is a B-tropic virus it will not be able to propagate efficiently in N-type cells and the mouse is unlikely to become viraemic. Inbred strains of mice with a high spontaneous incidence of leukaemia and viraemia, were found to contain at least one inducibility allele and to be susceptible to their own endogenous virus (ROWE and HARTLEY, 1972; STEPHENSON and AARONSON, 1972b).

In chickens, as mentioned above, susceptibility to infection of the endogenous viral subgroup depends on two alleles: the presence of the dominant receptor allele and the absence of an epistatic inhibitor gene. Such a genotype will be readily susceptible to infection by endogenous virus, and should the virus be activated in one or a few cells it will rapidly spread throughout the tissues of the host. Thus the expression of endogenous virus at the organism level depends on the ability of the activated

virus to spread by infection. Since the epistatic inhibitor locus is probably identical to the *gs* locus (see *I.*E. above), partial expression of the viral genome may inhibit spreading infection and thus be of selective advantage to the host.

There is evidence from studies with mammary tumour viruses too for genetic control of activation and of susceptibility to infection (BENTVELZEN *et al.*, 1972). As in chickens, partial expression of the endogenous genome may cause resistance to exogenous infection (DAAMS *et al.*, 1968; BENTVELZEN *et al.*, 1970). If this phenomenon is generally true of oncornaviruses, one might postulate that endogenous viruses only become virulent and infectiously-transmitted in "foreign" host strains or species.

III. Epigenetic Regulation of Endogenous Viruses

Infectious oncornavirus particles serve as a remarkably good tool with which to investigate the expression of inherited oncornavirus genes. The virus particles represent extracellular packages which contain the viral genome, thereby providing a selective probe (by nucleic acid hybridization) to reveal the inherited DNA genomes and their RNA transcripts. Likewise, the proteins of the virus particles, which are antigenic, have been used to provide specific antisera with which to probe the gene products of the inherited viral genomes. As described in the foregoing section, these probes have been crucial in describing the genetic and epigenetics of endogenous viral genomes. If, as seems reasonable, we regard the inherited viral genes as cellular genes, these studies represent an interesting analysis of gene expression in eukaryote cells. Most of these studies, however, have been conducted with fibroblastic cultures or established cell lines. The inherited oncornavirus genomes must, of course, be represented in every cell of the organism, and there is some tissue specificity in their expression. For instance, expression of *gs* antigen in chicken embryos is greatest in visceral organs such as the liver and pancreas (DOUGHERTY and DI STEFANO, 1966; PAYNE and CHUBB, 1968). Differential expression of *gs* antigen is also observed in mice (HUEBNER *et al.*, 1970), and the complete endogenous virus of AKR mice appears late in embryonic development. (ROWE and PINCUS, 1972).

While leukaemia viruses replicate in many cell types, their oncogenic potential is restricted to certain haemopoietic cells. The genetically-transmitted leukaemia viruses of mice are associated with lymphomatosis, usually of T cells. The endogenous virus of chickens has not yet been shown to induce neoplasms of any kind. Mammary tumour viruses appear to be restricted to a few cell types for replication; following neonatal infection, antigens are found in haemopoietic cells but complete replication

occurs only in the mammary gland and in the epididymis (BENTVELZEN *et al.*, 1970), and tumours occur only in the mammary gland. Both the replication and the neoplastic expression of mammary tumour virus, whether genetically or infectiously transmitted is hormone-dependent (MÜHLBOCK, 1972). This may prove to be true of the leukaemia viruses too. We have very little understanding of the epigenetic control of the susceptibility of cells to neoplastic transformation.

IV. Conclusions

Viral carcinogenesis in mice and chickens is complex. It is mediated through viral genomes which may be inherited or acquired by infection. The oncornaviruses are themselves subjected to host genetic control, endocrine control, and activation by external agents such as carcinogens. Much of the work described here was only possible through the use of inbred strains of animals and of clearly defined experimental oncornaviruses. I believe the concepts illustrated here are relevant to human cancer (see SPIEGELMAN *et al.*, 1972), but the relative importance of the different factors in human carcinogenesis will be difficult to unravel.

The ubiquitous occurrence of inherited oncornaviruses and the frequent expression of oncornavirus genes during embryonic or post-natal development, suggests that these genomes might have a biological role to play that is not restricted to tumour induction (HUEBNER *et al.*, 1970; TEMIN, 1971).

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HERPES VIRUSES AND NEOPLASIA

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CONTENTS

Herpes viruses and neoplasia

I. Introduction

No fewer than eight herpes viruses have now been associated with neoplasia. The purpose of this contribution is to examine the strength of their candidature as aetiological agents. If we were to keep a completely open mind and ask how a virus can cause a tumuor, we can invent a large number of mechanisms both indirect and direct. There are many possible indirect mechanisms; for example, a virus might cause immuno-suppression and so invalidate the body's surveillance mechanisms. But such a mechanism, by itself, would be non-specific. Any one virus could induce many types of tumour by this means. It is contrary to the experience we have of other DNA and RNA oncogenic viruses. I shall therefore restrict consideration to direct mechanisms where the virus acts on a target cell which, as a result, undergoes malignant transformation.

How fitted are the herpes viruses to induce this type of cellular change? The question was discussed by ROIZMAN (1971). He pointed out that herpes viruses often infect productively; thus, the infecting virus particle enters the cell and induces a number of sequential operations leading to the formation of progeny virus particles. This train of events, which has been intensively studied in the laboratory, is invariably accompanied by the death of the herpes virus infected cell. It is therefore incompatible with tumour formation which, above all, requires cell multiplication. However, herpes viruses may also infect non-productively, in which case the genome evidently remains intact for long periods without forming progeny. Such non-productive infections have been recognized for many years in the guise of virus latency. The classical observations on zoster by HEAD and CAMPBELL (1900) provided the basis for supposing such a phase can exist and the recent ingenious experiments of STEVENS and COOK (e.g., 1972) substantiate the idea that the genome of herpesviruses can persist latently in a neurone. It is important to note

that this cell offers sanctuary to the virus genome with minimum constraint; since the neurone does not multiply there are many possible states in which the virus genome might persist. On the other hand, if a virus genome, or part of it, is to be represented in each and every tumour cell, it must multiply in step with the cell. The only certain way for this to occur is by integration in host cell chromosomes which may or may not require covalent linkage between virus and host cell DNA.

II. Association and Causation

It is not difficult to show an association between a virus and cancer. This can be done by sero-epidemiological investigation, detection of antigen in cells or any of the very sophisticated techniques now available for detecting virus genomes in cells. On the other hand, it is nearly impossible to show that a virus causes a tumour. No matter how sophisticated the technique is, detection of virus or virus product in a tumour cell can do no more than indicate an association.

The strict way to establish a causal relationship is to take a cloned purified preparation of the virus in question and show that this will cause cell transformation or tumours when the same material specifically inactivated will not do so. This is seldom possible with human material.

I shall discuss specific examples of herpes viruses which have been considered possibly oncogenic. The evidence will then be discussed and conclusions of a sort drawn.

III. Lucke's Virus

The renal adenocarcinoma of the leopard frog was the first tumour presumed to be caused by a herpes virus. About half these tumours were known to contain the type A intranuclear inclusion body characteristic of herpes viruses (Cowdry, 1934) and the tumours were shown to be transmissible by cell-free extracts (Lucké, 1934, 1938). Fawcett (1956) found that tumours containing these inclusions also contained virus particles morphologically like herpes virus. Since that time several workers have corroborated this observation (Lunger, 1964; Lunger *et al.*, 1965; Zambernard and Mizell, 1965; Zambernard *et al.*, 1966; Stackpole, 1969). There is no question that the virus called Lucké's virus is morphologically a herpes virus and that it is associated with inclusion-bearing tumours, no particles having been discovered in tumours not containing inclusions. There is evidence too, that Lucké's virus contains DNA (Zambernard and Vatter, 1966) and that the DNA is double-stranded with a guanine + cytosine content of 45-47% (Wagner *et al.*, 1970; Gravell, 1971).

The teasing problem for many years has been whether Lucké's virus is indeed the cause of the tumour. One of the difficulties has been that not all adenocarcinomas contain inclusions and virus particles. This has now been clarified. The important observation of ROBERTS (1963) that none of fifty-four tumours developed at 20°-24° C contained inclusions while all of seven developed at 5 °C did so, led eventually to the conclusion that the virus was temperature sensitive. RAFFERTY (1965) showed that inclusions developed in tumours which had previously been free of them when frogs were transferred to low temperatures. MIZELL *et al.* (1968, 1969a) showed the development of virus particles in fragments of tumour implanted into the eye chambers of animals maintained at low temperatures and BREIDENBACH *et al.* (1971) have observed the induction of herpesvirus particles in fragments of inclusions from tumours explanted in tissue culture at 7.5 °C. Finally, MIZELL (1971) now reports the detection of RNA in inclusion-free tumours which hybridizes with Lucké's virus DNA.

A second difficulty has been whether the oncogenic principle in cell-free filtrates that induce these adenocarcinomas is Lucké's or some other virus. Here confusion is considerable. Attempts to isolate Lucké's virus have resulted in a profusion of different agents, including cytoplasmic polyhedral viruses, a papovavirus and another herpes virus, FV4 (GRANOFF, 1971). FV4 was isolated by RAFFERTY (1965) but could not be shown to induce tumours in tadpoles, a result that has been confirmed by GRANOFF *et al.* (1969). GRANOFF (1971) quotes evidence that this virus is distinguishable from Lucké's virus by base composition of DNA, absence of homology by DNA-DNA hybridization and lack of serological relatedness. We are not at all helped by this profusion of virus isolates; indeed, each additional virus isolated tends to reduce our confidence that Lucké's virus is the aetiological agent of the carcinoma.

The difficulties are, to some extent, overcome by the experiments of TWEEDELL (1967) and by MIZELL *et al.* (1969b). TWEEDELL (1967) was able to show that cytoplasmic fractions of inclusion-bearing tumours containing virus particles and filtrates of these, when injected into *Rana pipiens* embryos gave rise to adenocarcinomas at metamorphoses. On the other hand, extracts of normal kidney or inclusion-free tumours did not have this property. MIZELL *et al.* (1969b) obtained a fraction from inclusion-bearing tumour material by ultracentrifugation. This fraction contained predominantly enveloped herpesvirus particles. When injected into embryos typical adenocarcinomas developed.

IV. Marek's Disease Virus

Marek's disease is an infectious and contagious disease of fowls characterized by lymphoproliferative changes with a predilection for nerve

tissue. There is a strong genetically determined predisposition to the disease (COLE, 1971). Marek's disease virus was first revealed by co-cultivating tumour, blood or liver cells with cultured chick kidney cells (CHURCHILL and BIGGS, 1967). Infectivity remained firmly cell associated (BIGGS and PAYNE, 1967) and provided that cells remained intact they reproduce the disease when inoculated into chickens. The cytopathic effect in tissue culture was characteristic of a herpes virus. Despite the earlier failures to detect cell-free infectivity, virus particles were demonstrated with the morphology of herpes virus (EPSTEIN *et al.*, 1968). Prolonged cultivation in tissue culture led to a change in plaque morphology, the loss of the "A" antigen and loss of pathogenicity (CHURCHILL, CHUBB and BAXENDALE, 1969). Parallel findings were made at about the same time by American workers (see NAZERIAN, 1971). NAZERIAN (1970) showed that some infective virus could be freed from tissue culture cells and it is now possible to detect free virus with relative ease (CALNEK, HITCHNER and ADLDINGER, 1970). Though infective virus cannot be directly detected in lymphoid cells, it is now clear that *in vivo* some cells do produce virus, notably those in the epithelium of the feather follicle (CALNEK, ADLDINGER and KAHN, 1970) and cell-free virus from this source produces the disease when injected into susceptible birds (NAZERIAN and WITTER, 1970), thus accounting for the high contagiousness of the disease. NAZERIAN (1971) summarizes the *in vivo* behaviour of the virus as follows: 1) most organs of the chicken become infected with virus covertly; no antigens or infective virus can be demonstrated; 2) in the bursa of Fabricius, kidney, lung and thymus, antigen and incomplete virus particles are occasionally detected; 3) in the feather follicle productive infection occurs and infective virus is released. In addition, there is some evidence that Marek's disease virus depresses both cellular and humoral immunological responses.

The most remarkable finding with Marek's disease is that it can be prevented by vaccination, either with attenuated virus (CHURCHILL, PAYNE and CHUBB, 1969) or with the non-pathogenic related virus of turkeys (OKAZAKI, PURCHASE and BURMESTER, 1970). The mechanism for this protection is still uncertain since it does not prevent superinfection by virulent virus or its shedding, seems not to be an interference effect and does not appear to be due to humoral or cellular immunity alone (for discussion see NAZERIAN, 1971).

The role of Marek's disease virus as an aetiological agent thus appears reasonably secure. In particular, prevention of the disease by vaccination either with attenuated virus or with a related virus reduces the probability that other agents might be responsible.

V. EB virus

Burkitt's lymphoma was described as a clinical entity (BURKITT,

1958) which, it subsequently transpired, had peculiar epidemiological features (BURKITT and O'CONOR, 1961). The tumour, which has a characteristic histological pattern (WRIGHT, 1963) occurs predominantly in African children in areas where the minimal temperature is at least 15 °C and where the annual rainfall is at least 20 inches (HADDOW, 1963). This generated the idea of a causal vector transmitted virus, a concept that was supported when it transpired that the disease is common in areas of Papua with similar climate (TEN SELDAM *et al.*, 1966). However, it is now recognized that the disease also occurs (rarely) in countries like the U.S.A. and Great Britain.

Attempts to find a causative agent were at first confused by the number of candidate viruses; BELL (1967) considered at least six. The Epstein-Barr (EB) virus came to light in an unusual fashion. EPSTEIN and BARR (1964) had established cultures of lymphoblastic cells from biopsies of Burkitt's tumours and made the important discovery (EPSTEIN *et al.* 1964) that a small proportion of the cells of some of these cell lines contained virus particles with the morphology of herpes virus. There followed a series of papers reporting the same phenomenon in other lines of EB cells (for refs. see BELL, 1967). The virus containing cells also contained specific antigens by immunofluorescence tests.

Development of the immunofluorescence tests enabled seroepidemiological studies to be made and it became clear that antibody to EB virus is widespread (HENLE and HENLE, 1966). These studies led in an unexpected direction and it is now certain that this virus causes classical infectious mononucleosis (DIEHL *et al.*, 1968; HENLE, HENLE and DIEHL, 1968; NIEDERMAN *et al.*, 1968; EVANS, NIEDERMAN and MCCOLLUM, 1969).

There are now the strongest indications that EB virus is associated not only with Burkitt's lymphoma but also nasopharyngeal carcinoma (DE THÉ *et al.*, 1969). There is a growing body of evidence of the aetiological role of EB virus in these neoplastic conditions which follows.

1) *Evidence of virus in biopsy material.* Though the search for virus particles and intracellular virus antigens in biopsies of Burkitt's lymphomas has generally proved negative, a virus specific membrane antigen (MA) can be detected in most cells of most biopsies (KLEIN *et al.*, 1967) and where this antigen was not detected, it was found that the cells were coated with host IgG. However, this has not yet been demonstrated with biopsies from cases of nasopharyngeal carcinoma. On the other hand, cultured lymphoblasts from both Burkitt's lymphoma and nasopharyngeal carcinoma show evidence of intracellular antigens in about 10% of the cells. Predominant amongst these was the socalled early antigen (EA), but some also had the capsid antigen (VCA) and virus particles (NADKARNI *et al.*, 1970). Stronger evidence is provided by efforts to detect

the viral genome in biopsy material. All Burkitt and nasopharyngeal tumour tissue tested have shown the presence of EB virus DNA using DNA-DNA hybridization or RNA-DNA hybridization, and have given estimates of several genome equivalents per cell (ZUR HAUSEN *et al.*, 1970; ZUR HAUSEN and HOLTHAUSEN, 1971; NONOYAMA and PAGANO, 1971).

2) *Evidence of viral transformation by EB virus.* When lymphocytes from 'normal' individuals are cultured, this generally results in failure to establish a permanent cell line. However, cells from acute infectious mononucleosis and Burkitt's and nasopharyngeal tumours regularly develop into permanent lymphoblastic lines (DIEHL *et al.*, 1968; NADKARNI *et al.*, 1970). Again, cells from apparently 'normal' people with antibodies against VCA may develop into permanent EB positive cell lines, suggesting that the virus genome persists in such cells (HENLE and HENLE, 1973). The establishment of these lines is accompanied by the emergence of EA and MA and sometimes VCA and virus particles. EB DNA can be detected by hybridization. The induction of viral antigens is enhanced by arginine deprivation (HENLE and HENLE, 1968), mitomycin C (YATA *et al.*, 1970) or halogenated pyrimidines (GERGELY *et al.*, 1971; GERBER, 1972; HAMPAR *et al.*, 1972). There is reason to believe that the complete genome is resident in all cells of these lines since after cloning under antibody cells still make virus antigens (MAURER *et al.*, 1970; ZAJAC and KOHN, 1970).

Attempts to transform cells from normal donors have been successful. This has been accomplished using x-irradiated EB carrying lymphoblasts and filtered or partly purified virus. It can be abolished by procedures designed to inactivate the virus (HENLE *et al.*, 1967; POPE *et al.*, 1969; GERBER *et al.*, 1969; NILSSON *et al.*, 1971). Except for one report where primary human fibroblasts were morphologically transformed (EPSTEIN, 1971), the target cell has been the human lymphocyte. Interestingly, there are two recent reports of the lymphocytes of non-human primates being transformed *in vitro* (WANER *et al.*, 1972; MILLER *et al.*, 1972).

It looks very much as though EB virus is an important factor (perhaps the only one) responsible for establishing continuous lymphoblast cultures. If this is so, these cultures may be said to have undergone viral transformation since they have altered morphologically, are not contact inhibited, appear 'immortal', have chromosomal aberrations, contain virus DNA and neo-antigens.

3) *Sero-epidemiological evidence.* The main evidence for the association of EB virus with infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma has been sero-epidemiological. As noted above, indirect immunofluorescent tests have contributed most infor-

mation. Lately, these tests have been considerably refined—mainly by the Henles so that it is now possible to distinguish different antigens (and hence antibodies) on the basis of the location and pattern of immunofluorescence in the cells examined and whether the antigen is early (not suppressed by cytosine arabinoside) or will agglutinate nucleocapsids. The following antigens are recognized: MA (by surface fluorescence), VCA (antibody to which agglutinates nucleocapsids), EA (found in presence of cytosine arabinoside). The last shows two characteristic patterns: either diffuse (D) or restricted (R). These antigens are undoubtedly complex and are not yet characterized physically. Their interest lies in the remarkable differences in the patterns of antibody titres found in sero-epidemiological studies. For example, a primary EB virus infection confers VCA antibodies probably for life. All patients with Burkitt's lymphoma or nasopharyngeal carcinoma possess these antibodies in high titre. However, the behaviour of antibodies to EA are of special interest. In infectious mononucleosis 70% of patients have transitory anti-EA (D), the presence of which reflects the severity of the disease. Nasopharyngeal carcinoma patients show 85% anti-EA (D) with low and less frequent titres of EA anti-(R). On the other hand, Burkitt's lymphoma patients show 85% anti-EA(R) with low titre only of anti-EA (D). The titres of these antibodies also appear to have prognostic significance. High anti-EA titres indicate a poor prognosis as does the decline in anti-MA (see HENLE and HENLE, 1973, for references). Though these differences in antibody pattern are, as yet, wholly unexplained, they appear to have some relationships with the patterns of disease which could scarcely be the case were EB virus a mere passenger.

The case for ascribing an aetiological role to EB virus in Burkitt's lymphoma and nasopharyngeal carcinoma is strengthening. The virus exhibits many characteristics of a potential oncogen. However, the epidemiological pattern which curiously first suggested a virus aetiology for Burkitt's lymphoma, does not fit a ubiquitous parasite like EB virus which infects a major portion of the world's population. It is very possible that a co-factor is necessary. Indeed, BURKITT (1969) suggests that chronic intense malarial infection might be such a factor and KAFUKO and BURKITT (1970) show close epidemiological coincidence between falciparum malaria and Burkitt's tumour.

In summary, there is little doubt that EB virus is the cause of classical infective mononucleosis. But its candidature for an aetiological role in Burkitt's lymphoma or nasopharyngeal carcinoma is still uncertain. Despite the evidence that the virus genome is present in all tumours tested, the evidence of *in vitro* transformation and the seroepidemiological evidence we are wholly without evidence that the virus will cause lymphomas in man.

VI. Some Other Herpesviruses

During the past few years, the list of herpes viruses has grown considerably. Some of these are associated with neoplastic processes. This section deals briefly with some of them.

A. Herpesviruses of Primates Other than Man

The first oncogenic simian herpesvirus to be isolated was *Herpesvirus saimiri* (MELÉNDEZ *et al.*, 1968) isolated from a spontaneously degenerated culture of squirrel-monkey kidney. It is evidently a common latent parasite of these monkeys since 50-70% possess antibody to it. It multiplies well in a number of simian cell lines and the cells of a few other species. The morphology is typical of a herpes virus (MORGAN *et al.*, 1970) and it contains double-stranded DNA with a G+C content of 69% (GOODHEART, 1970). The virus is serologically distinct from all of many other herpes viruses tested (cf. MELÉNDEZ *et al.*, 1971). The virus is of interest because it remains latent in its own host, the squirrel monkey, in which it produces no recognizable disease of any sort. However, when inoculated into cotton-top marmosets, owl monkeys, cinnamon ring-tail monkeys, African green monkeys, black spider monkeys or rabbits, a malignant lymphoma results. On the other hand, no disease has been produced in macaques, baboons or chimpanzees (see MELÉNDEZ *et al.*, 1971). A second similar virus, *Herpesvirus ateles* (MELÉNDEZ *et al.*, 1972) behaves similarly in that it produces no disease in its own host but malignant lymphomas in other species. DIENHARDT, FALKE and WOLFE (1973) draw attention to biological similarities between these two viruses and EB virus in man for while there is no infective virus or antigen detectable in neoplastic cells *in vivo*, their cultivation leads to the prompt appearance of virus and antigens within 1-3 days (FALKE *et al.*, 1972). Again, as with EB virus, permanent cell lines contain a proportion of degenerating cells producing virus and virus antigens.

B. Cotton-tail Herpesvirus

A new herpes virus was isolated from cotton-tail rabbits by HINZE (1971a) and shown by him to induce lymphoproliferative disease (HINZE, 1971b). The majority of rabbits respond to infection with a benign lymphoid hyperplasia but in a proportion the disease progresses to a malignant lymphoma. The virus seems to be host-specific but will multiply in cells derived from *Oryctolagus* as well as *Sylvilagus*, giving a classical cytopathic effect (HEINE and HINZE, 1972). Experimental infection of

cotton-tail rabbits leads to a chronic low-grade viraemia with lymphoproliferation. Where the disease progresses, there is characteristically infiltration of the viscera with immature proliferating lymphoid cells. No inclusions or evidence of cell destruction has been found *in vivo*. However, virus has been isolated from the leucocytes and a small proportion show the presence of virus antigen by immunofluorescence (HINZE and WEGNER, 1973). Attempts to establish permanent lymphoblast lines *in vitro* have so far failed, but antigen containing cells and infective virus have been demonstrated in shor-term cultures.

C. Guinea-pig Herpesvirus

HSIUNG and KAPLOW (1969) isolated a herpesvirus from a spontaneously degenerating kidney cell culture from leukaemic guinea pigs and subsequently from non-leukaemic animals, though it was rare in leukaemia-resistant strains (HSIUNG, KAPLOW and BOOSS, 1971). The isolation depends on culturing (or co-cultivating) whole cells. In culture typical cytopathic effect was found and particles of characteristic herpes morphology could be demonstrated. However, no particles have been seen in leukocytes taken *in vivo*. Experimental infection of Hartley guinea pigs resulted in persistent infection which lasted at least two years. The spleen appeared to be the primary site of persistence. No disease was produced. Finally, HSIUNG, FONG and GROSS (1973) report *in vitro* transformation of hamster cells by this virus. HSIUNG *et al.* (1973) point out that C-type particles have always been observed in the tissues of animals with leukaemia while herpesvirus particles have not. On the other hand, the herpes virus is constantly reactivated on cultivation of spleen cells while C-type virus can be reactivated with special measures. In such experiments they have found cells doubly infected with both viruses judged by electron microscopy and go on to speculate that C-type virus may be an essential co-carcinogen.

D. Sundry Observations

MACKAY (1969) reported a herpes virus in macrophage cultures made from sheep pulmonary adenomatosis lesions. There is slight evidence that the virus is implicated in the pathogenic disease. MACKAY and NISBET (1971) report the successful transmission of the disease by filtrates of lesions and of first tissue culture passed material with subsequent isolation months later of the herpesvirus. The evidence is, however, still uncompelling and further work needs to be done.

KARPAS and SAMSON (1971) reported the induction of a malignant lymphoma in the Syrian hamster by equine herpes virus 3. Hamster embryo cells were transformed *in vitro* by the virus and formed tumours when inoculated into new-born animals. However, the relationship between the lymphoma and the virus has not been satisfactorily established.

VII. Genital Herpes Simplex Virus

Carcinoma of the uterine cervix has been the object of scrutiny for a great many years. It is an example of a malignant tumour which exhibits recognizable precancerous changes and which has some definite epidemiological features. At a recent symposium, ROTKIN (1973) reviews the epidemiological features, some of which, he points out, were established more than 100 years ago. In his analysis, he emphasizes two principal co-variates of the disease —multiple sexual partners and age of first intercourse. At present, the most likely agent is herpes simplex virus type 2.

The recent interest in genital herpes in relation to carcinoma of the cervix seems to have begun with the observation (NAIB, 1966) of four cases in which herpes infection co-existed. At about the same time, differences were established between oral (type 1) and genital (type 2) isolates of herpes virus (SCHNEWEIS, 1962; PLUMMER, 1967; NAHMIAS and DOWDLE, 1968) and this has enabled sero-epidemiological surveys to be made which seem, in general, to point to an association between carcinoma of the cervix (and its precursor conditions) and past infection with herpes virus type 2 (e.g., NAIB *et al.*, 1966; RAWLS *et al.*, 1968; SKINNER, THOULESS and JORDAN, 1971). However, there are a number of difficulties in the interpretation of these studies. First, the antigenic constitution of the two types of virus are similar. Both structural and non-structural antigens have common as well as type specific determinants (see WILDY, 1973). Secondly, both viruses are ubiquitous so that the majority of human sera will react with one or both type specific antigens. Thirdly, the time interval between infection and the first indication of disease is long. Fourthly, there is controversy as to whether the two types form discrete clusters or if intermediate strains exist. These questions are discussed by PLUMMER (1973) and RAWLS (1973).

Other evidence using human material has been reported by AURELIAN, ROYSTON and DAVIS (1970) who found herpes virus type 2 antigens in exfoliated cervical tumour cells while normal cells from the same patient had none. AURELIAN (1973) now reports the presence of a particular virus specific antigen (AG4) which she detected in 90% of carcinomas and in only 10% of controls.

Attempts to detect herpes virus genetic material in thirteen carcinomas by DNA-DNA and RNA-DNA hybridization have been unsuccessful using the techniques which succeed for EB virus (ZUR HAUSEN, 1973). However, ROIZMAN and FRENKEL (1973) using reassociated hybridization kinetics (cf. FRENKEL and ROIZMAN, 1972) report that a cervical tumour contained RNA transcripts complementary to 5% of the total herpes type 2 genome and further, that type 2 virus DNA representing only about 40% of the genome, was detected which appeared to be covalently linked to the cell DNA, there being of the order of one copy per cell. These studies indicate that herpes virus type 2 genetic material is present and partly expressed in tumour tissue. The last, which represents work on only one tumour, also suggests that part of the herpes genome can integrate in cell chromosomes.

There have been a number of attempts to transform cells with herpes virus type 2 and to induce tumours in animals. The problem is to devise a biological system which restricts productive infection and cell killing. This has evidently been achieved by the use of ultraviolet irradiation. DUFF and RAPP (1971), using heavily irradiated type 2 virus, produced morphological-transformation of primary hamster cells. Herpes specific antigens were present in a proportion of these cells and the cells readily caused tumours when injected into new-born hamsters. Similarly, MUNYON *et al.* (1971) were able to introduce a herpes specified thymidine kinase gene into a line of cells lacking that enzyme. RAPP and DUFF (1973) now report an extension of their work. They have successfully transformed hamster embryo fibroblasts with type 1 herpes virus (2/12 strains) and type 2 virus (7/15 strains). One transformed cell line induced by type 2 virus was oncogenic; two other lines tested were not. Neutralizing antibodies were found in hamsters bearing tumours. Attempts to immunize weanling hamsters with either type of herpes virus did not prevent tumour formation but curiously enhanced metastasis.

Cell transformation has also been attempted using supra-optimal temperatures to restrict productive infection. DAVAI and MUNK (1972) infected human embryo lung cells with a syncytium forming variant of herpes virus and held the cultures at 43 °C for a period. Cells were then allowed to grow up at 37 °C. The procedure was repeated twice more and the cultures which resulted contained many syncytia and virus antigens though no infective virus was usually found. This cell line has now been propagated through many passages: it occasionally "breaks down", giving infective virus. It has not yet been tested for oncogenicity.

There have been several attempts to induce tumours in animals with herpes viruses. Cervical infection of large numbers of mice with type 2 virus has resulted in a few tumours (NAHMIAS *et al.*, 1971; MUÑOZ, 1971). NAHMIAS *et al.* (1970) reported development of sarcomas in 7/541 hamsters surviving herpes type 2 infection. A large scale investigation is

now in progress using cebus monkeys which react to herpes infection somewhat like humans. Useful results can be expected in 3-5 years' time (SEVER, 1973).

In summary, there is sero-epidemiological evidence that herpes simplex virus type 2 is associated with carcinoma of the cervix. There is evidence that the genome (or parts of it) of this virus can integrate in mammalian cells causing transformation. Though the evidence for the integration of this virus genome in carcinomatous tissue is contradictory, the recent detection of a partial genome (and of specific herpes messenger RNA) in a tumour contributes powerfully to the idea that a part of the virus genome may integrate in human tissue. We still lack the direct evidence that we wish for and must await the demonstration that the virus can under controlled conditions cause carcinomas in primates (if not in man).

VIII. Discussion and Conclusions

The work reviewed above raises tantalising questions but can only lead to tentative conclusions. Except with Marek's disease and the monkey viruses we cannot claim that any herpes virus causes neoplasia.

There is no biological subgroup of oncogenic herpes viruses. Herpesviruses vary considerably in some of their biological and clinical properties. Among the most striking differences is the G+C content of the nucleic acid. In most other organisms (including viruses) biological groups have rather similar G+C contents while the herpesviruses span the range 35-75. The eight "oncogenic" herpes viruses span this range fully, Lucké's virus and the *Saimiri* virus illustrating the point.

There are, however, some common features amongst these candidate herpes viruses. The table summarizes as far as possible the data reviewed here. All the viruses commonly infect their host species. All appear to give rise to chronic persistence or latency. Most can be reactivated when appropriate cells are removed from the host and planted *in vitro* with or without other cells. Lucké's virus is exceptional in that temperature alone appears to control activation.

The possibility that latency indicates lysogeny as suggested, for example, by MIZELL and ZAMBERNARD (1965), must remain open. The evidence for this is still poor since lysogeny implies covalent integration of virus DNA in host cell DNA and though resident virus DNA has been detected in several examples, this does not necessarily indicate integration. Similarly, though *in vitro* transformation by other oncogenic viruses has been in several instances shown to be associated with integration, one can invent alternative explanations. The only virus for which there is such evidence is herpes simplex (ROIZMAN and FRENKEL, 1973) and that is in

material derived from one tumour. However, the phenomena of reactivation found with all these viruses are most easily explained in terms of the derepression seen when lysogenic viruses are induced.

Three herpes viruses can now be said to cause neoplasia. Marek's disease has been shown to be caused by cell-free virus and the disease can be prevented by vaccination with attenuated virus (or a related virus). The two monkey viruses regularly induce lymphomas in related (but not own host) species of monkey. EB and Lucké's virus probably cause neoplasia, but here we require formal proof—the lack is a suitable tissue culture system in which virus can be cloned. However, in both systems evidence is strong that the virus play an aetiological role.

The role of co-factors is interesting. In two instances, (Marek's disease and nasopharyngeal carcinoma), there is evidence that genetic factors play a major part in the emergence of disease: this may be so in other instances as well. With Burkitt's lymphoma it is very obvious that the virus can only play a causative role if there is some co-factor. Very possibly this is immunological.

So far I have been very restrained in discussing the role of herpes viruses in neoplasia. Though it is important to be critical and to demand hard evidence, this must not preclude imagination. Though hinting at it, I have not yet unleashed the argument of analogy, the mainspring of inductive biological thought. A glance at Table I shows considerable homogeneity in the behaviour of six of the eight herpes viruses. All except those of Lucké and herpes simplex are concerned in lymphoproliferative

Table I. Behaviour of eight herpes viruses.

	Ubiquity of infection	Lympho-proliferation	*In vitro* transformation	Tumours induced by filterable agent	Latent in host	Reactivable on culture (or co-culture of cells)	Evidence of resident genome	Evidence of integration	Causative-relationship
Marek	+	+	+	+	+	+	?	−	+
EB	+	+	+	?	+	+	+	−	−
Saimiri	+	+	+	+	+	+	?	?	+
Ateles	+	+	+	+	+	+	?	?	+
Cotton-tail	?	+	−	?	+	+	?	?	?
GP	?	+	+	−	+	+	?	?	−
Lucké	+	−	−	+	+	+	+	−	−
HS	+	−	+	?	+	+	+	+	−

processes. The temptation to summate the information about these six viruses is almost irrestible; if it is done, the message clearly is that these sorts of herpesviruses cause neoplasia. When we go that far, we are obliged to explain why we do not include the remaining two candidate viruses.

Let us preserve a sane approach. Analogy is not evidence and cannot substitute for it. Until we have evidence our critical appraisals must endure—only Marek's disease virus and two monkey viruses have been shown to cause neoplasia. However, analogy can and should be allowed to point the way, encouraging us to investigate the other herpes viruses.

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Electron micrograph, kindly supplied by Professor Joseph L. Melnick, of a human adenovirus which contains a DNA genome. The virus is 65 nanometers in diameter and contains 252 capsomers. The virus possesses icosahedral symmetry. The outline of the virus often appears hexagonal; and triangular phases each containing 21 capsomers may be seen. Over thirty antigenic types are known and many have proven to be oncogenic in hamsters.