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Animal and Plant Viruses with Double-Stranded Ribonucleic Acid

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ANIMAL AND PLANT VIRUSES WITH DOUBLE-STRANDED RIBONUCLEIC ACID

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ABSTRACT

Reoviruses occur widely in the respiratory and enteric tracts of man and animals, but their relation to disease is not clear. The present study concerns the physical-chemical characteristics and interaction with cells of reovirus type 3. A main objective of the study is to relate some of the distinguishing biological characteristics of the virus to the structure of its RNA.

Reoviruses are medium-sized, icosahedral viruses with a diameter of 75 mμ, and they contain RNA. The protein coat of the virus is composed of either 5- or 6-sided prismatic subunits, 92 in all. Each structural subunit of the protein coat is 75 A wide and possesses a hollow center 30 to 40 A in width and walls 15 to 20 A thick.

Among the three immunological types of reovirus, all type 3 strains agglutinate bovine erythrocytes, and some agglutinate human erythrocytes. None of the strains of reovirus types 1 and 2 agglutinate bovine erythrocytes, whereas they all agglutinate human erythrocytes. The characteristics of reovirus type 3 interaction with receptor substances set it apart from all other viruses. The reactive groupings on reovirus 3 are sensitive to sulfhydryl reagents; they are also sensitive to trypsin, but resistant to chymotrypsin; they have the ability to react with neuraminic acid-containing glycoprotein receptors, but lack neuraminidase activity.

The reproductive process of reovirus 3 is characterized by three features which distinguish it from other RNA viruses: 1. the multiplication of reovirus is relatively slow; 2. the inclusion which develops in reovirus-infected cells stains orthochromatically greenish-yellow with acridine orange, as if it contained DNA, yet it contains RNA; and 3. the multiplication of reovirus is inhibited by actinomycin D.

Further evidence of the unusual characteristics of reovirus was obtained in studies of the synthesis of DNA, RNA, and proteins in infected and control cells. During the viral growth cycle, cellular DNA synthesis is specifically inhibited, whereas RNA and protein syntheses continue at normal levels.

All of these results suggested that reovirus RNA may possess dis-

tinguishing structural features. The anomalous staining with acridine orange raised the possibility that reovirus RNA, unlike any other known RNA, may be double-stranded. Results of physical-chemical studies have provided strong evidence to support this view. Purified reovirus contains 14.6% RNA and no DNA. The sedimentation coefficient of the virus, $s_{20} =$ 630 S, is consistent with a minimum particle weight of 70×10^6, and a minimum complement of RNA of 10×10^6 molecular weight units per particle. The base ratios are complementary. The mole percent of G + C is 39.8. Reovirus RNA melts sharply with the T_m at 99°; it reacts minimally with formaldehyde at 32°; and is insensitive to the action of pancreatic ribonuclease. Thus reovirus RNA appears to have a secondary structure similar to that of DNA, namely a double-stranded helix. Both in this respect, and also with respect to the large amount of genetic material in the virus particle, reovirus is unique among animal viruses containing RNA.

There is a second virus, the wound tumor virus, that has the same size and structure as reovirus. This plant virus produces tumors in sweet clover and other plants and is transmitted by an insect vector in which host it also multiplies. Wound tumor virus also has a ribonucleic acid component which melts sharply at a high temperature, and therefore may be a double-stranded helix.

Tumor viruses so far studied all contain a relatively large amount of genetic material. As a repository for genetic information, the double-stranded helical structure may possess the necessary stability to insure continuity of the virus genome. All DNA tumor viruses have been shown to be double-stranded; all RNA tumor viruses may also be double-stranded.

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I. INTRODUCTION

INTRODUCTION

Reoviruses, formerly known as ECHO 10 virus, occur widely in man and animals (Sabin, 1959, Stanley, 1961, and Rosen, 1962). The letters r, e, and o in the term reovirus stand for respiratory, enteric, orphan. These agents were thought of as orphans because they could not be associated with a disease picture in man (Stanley, 1961 and Rosen, 1962).

The first reported isolation of a virus belonging to this group was by Stanley et al. in 1953 in Australia. They recovered an agent from a fecal extract obtained from an aboriginal child mildly ill with bronchopneumonia. This agent when inoculated into newborn mice produced an unusual illness manifest by retarded growth, jaundice, encephalomyelitis, oily fur, alopecia, and subsequent death. They named the agent hepatoencephalomyelitis virus. This was subsequently shown to be related to reovirus type 3.

In an independent study to determine the agents which were present in the human intestinal tract in health and in disease, M. Ramos-Alvarez and Sabin, 1954, found that in addition to the enteroviruses already known, i.e. polio and Coxsackie, there were present a number of other viruses which could be classified into distinct groups. Amongst these groups, there was one which could be easily distinguished not only from the other groups, but also from polio and Coxsackie viruses by a number of very characteristic features (Sabin, 1959). The viruses in this group were larger, 72 mμ as compared to 28 mμ; they possessed a much broader host range; they produced a viral inclusion in infected cells; and they possessed a hemagglutinin which reacted with receptor sites on human erythrocytes different from those of the other enteroviruses.

In 1959, Sabin called this group of viruses, reoviruses, to indicate their respiratory or enteric origin. It was possible to segregate all the strains known at the time into three distinct serological groups (Rosen, 1960). All the various strains subsequently isolated have been found to be related to one of the three prototype strains (Rosen, 1962). Although there is no reliable evidence that reoviruses can cause disease in man, it is clear that they are widely distributed in the human population. Examination of sera of patients in a Boston hospital, has shown, for example, that

antibodies against reoviruses are found with increasing frequency through adolescence and in more than 60 percent of persons over sixty years old (Lerner et al., 1962).

In experimental animals reoviruses cause a variety of disease pictures. They have been associated with an epidemic of coryza in chimpanzees in the laboratory (Sabin, 1956); with meningitis and necrosis of the choroid plexus when inoculated intracerebrally in monkeys (Hull et al., 1956 and Hull et al., 1958); and with lesions in the brain, heart, and liver when inoculated parenterally into newborn or suckling mice in large doses (Stanley et al., 1953, and Rosen et al., 1960). It has also been shown that the infection can be transferred from inoculated newborn mice to their litter mates (Stanley et al., 1953).

The only observations on the natural history of reoviruses in animals have been on three dairy herds in Maryland (Rosen, 1963). At the end of the first year of life, every calf had been infected with at least one of the serotypes of reovirus although no illnesses were observed which could be attributed to reovirus infection. The only evidence of infection was isolation of the viruses from animals or a serologic response to one of the three prototype strains.

The ubiquity of reoviruses in animal species is indicated by the fact that antibodies against these viruses have been found in cattle on relatively isolated islands in the South Pacific, and also in the sera of monkeys shortly after their capture in the forests of Panama (Rosen, 1962). The viruses isolated from animals have possessed no special features to distinguish them from those isolated from humans.

Only a few reports have appeared concerning the physical-chemical properties of reoviruses, their multiplication, and cytopathic effects. In the present study are described some of the structural characteristics of reovirus 3, Dearing strain, and a variant of the Dearing strain isolated in the laboratory; some biological and biochemical features of the protein coat of reovirus type 3 and its interaction with receptors on erythrocyte surface and in solution; the dynamics of multiplication of this virus in an established line of mouse fibroblasts; the effects of this virus on the synthesis of macromolecules in these cells; the secondary structure of the

nucleic acid of reovirus; and some preliminary studies on the physical-chemical properties of a plant virus, wound tumor virus.

II. GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

Growth medium for cells. L cells were grown in Eagle's minimum essential medium (MEM) (Eagle, 1959) supplemented with 5% horse serum. During the last 30 passages, 5% fetal calf serum (Colorado Serum Co. or Microbiological Associates, Inc.) was used instead of horse serum.

Growth medium for viruses. The virus seeds were grown in Eagle's MEM in which the concentration of amino acids was increased two-fold and that of vitamins four-fold. The concentration of sodium bicarbonate was 3.7 g/l, of ferric nitrate 0.1 mg/l, and of sodium pyruvate 0.11 g/l. This reinforced Eagle's medium was supplemented with 5% fetal calf serum.

Adsorption media for viruses. In virus growth experiments, the adsorption medium was that in which the particular virus stock had been prepared. For plaque assay, dilutions of virus were made in Eagle's MEM without serum, except where stated otherwise.

All media contained penicillin (500 units/ml), streptomycin (0.1 mg/ml), and mycostatin (25 units/ml).

Viruses. Reovirus type 3, Dearing strain, was obtained from Dr. H.J. Eggers as the 10th monkey kidney cell passage. The virus had been originally isolated by Dr. M. Ramos-Alvarez in Cincinati, Ohio. The virus was passaged three times in L cells in reinforced Eagle's MEM supplemented with 5% horse serum. The titer of the third passage was 1.9×10^8 PFU/ml. Seed virus was prepared by inoculating 0.3 ml of the above third passage material on complete monolayers of L cells in 60 mm plastic petri dishes (Falcon Plastics Company, Los Angeles). Adsorption was allowed to take place at 37° for 2 hours in a humidified atmosphere of 5% CO_2 in air, and virus growth medium, 5 ml per plate, was then added. Twenty-six hours later, the cells and supernate were collected, frozen and thawed three times, cellular debris removed by centrifugation, and the supernate was stored at -28° C. The titer of the Dearing strain seed was 2.5×10^8 PFU/ml.

A variant of reovirus 3, Dearing strain, was obtained after repeated passage of the original Dearing virus in L cells. Seed virus preparations of the variant strain were prepared and stored as described above; they contained $1.2 - 5.1 \times 10^8$ PFU/ml.

Immune sera. Immune serum against reovirus 3, Dearing strain, was prepared by injecting rats intraperitoneally with 0.5 ml of stock virus containing 10^6 - 10^7 infectious units and collecting serum one month later. Serum was inactivated at 56° C for 30 minutes. Immune serum against the Dearing virus variant was prepared by inoculating 19-day-old weanling mice with 0.2 ml of a suspension containing 3 x 10^6 PFU of virus. Two weeks later the mice were reinoculated intraperitoneally with 3 x 10^7 PFU. Serum was collected two weeks after the second inoculation and inactivated at 56° for 30 minutes.

Cross-neutralization titrations were carried out with reovirus 3, Dearing strain, and its variant. The results of the neutralization titrations demonstrated that antisera against the original or variant strain neutralized the original Dearing virus to a greater extent than the variant strain. Apart from the difference in sensitivity to antibody, the cross neutralization tests revealed no significant differences in the antigenic constitution of these viruses.

Similar observations have been previously made with influenza (Choppin and Tamm, 1960) and ECHO viruses (Karzon et al., 1959).

Cell cultures. L cells, strain 929, obtained from Microbiological Associates, Inc., were cured of PPLO infection with kanamycin (Pollock et al., 1960), and grown in 32-ounce prescription bottles at 37° under 5% CO_2 in air until complete monolayers formed. The cells were dispersed with 0.125% trypsin dissolved in Ca^{++} and Mg^{++} deficient phosphate buffered saline with added glucose (Dulbecco and Vogt, 1954). The cells were harvested, collected by centrifugation, and suspended in Eagle's MEM containing 5% fetal calf serum. Plates for growth curve experiments were seeded with 5 x 10^5 cells, and those for plaque assays received 1 x 10^6 cells per plate. The cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Monolayers for plaque assay were complete in three days and were used within the next 24 hours.

Diluents. Buffered saline (BS) consisted of 0.85% NaCl buffered at pH 7.2 with 0.01 M phosphate. The solution designated phosphate-buffered saline (PBS) contained 0.0081 M KH_2PO_4, 0.0015 M Na_2HPO_4, 0.137 M NaCl, 0.0027 M KCl, 0.0009 M $CaCl_2$ and 0.0005 M $MgCl_2 \cdot 6H_2O$ (Dulbecco and Vogt, 1954). Calcium borate buffered saline (CaBBS) contained 8.50 gm NaCl,

1.0 gm $CaCl_2$, 1.203 gm H_3BO_3, 0.052 gm $Na_2B_4O_7 \cdot 10H_2O$ and 1000 gm of deionized distilled water. The pH was 7.03.

Erythrocytes. Chicken, guinea pig, and human O cells were obtained by venipuncture and washed three times with BS. Goose cells were provided by Dr. Casals. Ox, sheep, and rabbit cells were obtained from Cappel Laboratories as stabilized suspensions. Sheep erythrocytes were sometimes obtained from animals maintained at The Rockefeller Institute. The erythrocytes were suspended in BS to a final concentration of 0.25% and were used within two days.

Plaque assay. Ten-fold dilutions of viruses were prepared in Eagle's MEM. Complete monolayers of L cells in petri dishes were inoculated with 0.3 ml of the appropriate dilutions of virus, and incubated at 37° in 5% CO_2 in humidified air. Two plates were used for each dilution. During the adsorption period of 2 hours for reoviruses and 1 hour for vaccinia the inoculum was redistributed every thirty minutes. After adsorption was completed, each plate received 10 ml of an overlay medium consisting of equal volumes of 1.8% Bacto-Difco agar and two times concentrated reinforced Eagle's MEM containing 10% fetal calf serum. Fetal calf serum proved superior to horse serum; the plaques could be enumerated sooner because of their larger size. After incubation for 6 days, 5 ml of a second overlay containing 0.005% neutral red was added to each culture. The plaques, which were morphologically indistinguishable for Dearing virus and the variant strain, were counted 12-18 hours later.

Hemagglutination titrations. Test tubes with hemispherical bottoms and an internal diameter of 10 mm were used. Serial two-fold dilutions of virus were made in 0.3 ml BS, and then 0.3 ml of BS was added to each tube followed by 0.6 ml of a 0.25% suspension of erythrocytes. The tubes were held on a stand at 4° C for 3 hours, and then examined from below with a mirror so as not to disturb the agglutination patterns. The endpoint was taken as the highest dilution of virus causing partial (2+) hemagglutination, and the titer expressed as the reciprocal of dilution at endpoint. It was considered that one hemagglutinating unit (HAU) of virus was present at the endpoint.

III. RESULTS

A. Structure and Reactive Sites of Reovirus Type 3, and Virus Interaction with Receptor Substances

Structure and Reactive Sites of Reovirus Type 3, and Virus Interaction with Receptor Substances

Reoviruses have been found to be about 72 mμ in diameter, as determined by filtration through gradocol membranes (Sabin, 1959); to be ether resistant (Sabin, 1959; Rosen et al., 1960), indicating that a peripheral structural lipid was not a necessary component for biological activity (Franklin, 1962a); and to possess a hemagglutinin for human group O erythrocytes, but not for chicken, guinea-pig, sheep, or bovine erythrocytes (Sabin, 1959). The hemagglutinating activity is inhibited by specific antibodies (Goldfield et al., 1957). The fact that treatment of human erythrocytes with V. cholerae filtrate did not affect hemagglutination by reovirus type 2 (Dardanoni and Zaffiro, 1958) indicated that the reovirus receptors on human erythrocytes were probably distinct from myxovirus receptors. That they were also different from those involved in hemagglutination by ECHO viruses was shown by the finding that potassium periodate rendered human erythrocytes inagglutinable by reovirus, but had no effect on ECHO virus hemagglutination (Dardanoni and Zaffiro, 1958).

In this section, some details of the reovirus structure will be analyzed; agglutination of bovine and sheep erythrocytes by reovirus 3 at 4° will be described; evidence will be presented that a viral protein possessing sulfhydryl groups appears to be essential for reovirus 3 hemagglutinating activity and infectivity; and that reovirus 3 is capable of reacting with neuraminic acid-containing receptors on bovine erythrocytes and in solution.

Materials and Methods

Viruses. Thirty-two strains of reovirus types 1, 2, and 3 were obtained from Drs. L. Rosen, A.B. Sabin, E.H. Lennette, J.B. Nelson, and A.M. Lerner, and are listed in Tables III and IV. The viruses were grown in monkey kidney cells and the cell-associated and released virus harvested when cytopathic effects were maximum. The viruses were used after 2 or 3 passages in this laboratory.

The Amy strain of reovirus type 2 was obtained from Dr. Hans J. Eggers as the 10th monkey kidney cell passage, and was passaged once in monkey kidney cells.

A concentrate of influenza B virus, Lee strain, was prepared by Dr. Purnell W. Choppin as follows: infected allantoic fluid was centrifuged at 600 g for 10 minutes and the sediment discarded; the supernate was centrifuged at 40,200 g for 1 hour and the pellet resuspended in phosphate buffered saline. The hemagglutination titer of the suspension was 20,480. The reovirus seeds were stored at -28° and the influenza virus concentrate at -55°.

Cell culture. Primary cultures of rhesus monkey kidney cells in tubes were obtained from Microbiological Associates, Inc.

V. cholerae filtrate. The Inaba strain of V. cholerae was grown in neopeptone broth, and a filtrate was prepared according to Tyrrell and Horsfall (1952). Treatment of chick red blood cells for 1 hour at 37° with the filtrate diluted 1:64 rendered the cells inagglutinable by 4 HA units of Lee virus.

Trypsin. Sterile, 3x crystallized, lyophilized trypsin was obtained from Worthington Biochemical Corporation, Freehold, N.J.

Chymotrypsin. Alpha Chymotrypsin (Worthington Chymotrypsin I), 3x crystallized, was obtained from Worthington Biochemical Corporation, Freehold, N.J.

p-Chloromercuribenzoic acid, obtained from Mann Research Laboratories, Inc., New York. A 10^{-3} M stock solution was prepared as described previously (Choppin and Philipson, 1961).

Reduced glutathione, obtained from Mann Research Laboratories, Inc., New York, was dissolved in water and the pH adjusted to 7.2 with 0.1 N NaOH.

Genetron 113. Genetron 113 was obtained from the General Chemical Division of Allied Chemical, New York, N.Y.

Infectivity titrations. Serial 10-fold dilutions of virus were made in Eagle's MEM. Tube cultures of monkey kidney cells were washed twice with Eagle's MEM, 1.9 ml of Eagle's MEM was added, the tubes were gassed with 5% CO_2 in air, and 0.1 ml of diluted virus was inoculated. The tubes were rolled at 37° C and were examined for development of viral cytopathic effects. The final reading was made on the 8th day, and the 50% infective end-point calculated.

The plaque procedure which employs monolayers of L cells was suitable for several, but not for all of the viruses examined.

Hemagglutination Inhibition Titrations. Serial two-fold dilutions of inhibitor were made in BS or CaBBS in 0.3 ml volumes. Appropriately diluted virus was then added in 0.3 ml volumes and the mixtures incubated for 1 hour at 37° after which 0.6 ml of 0.25% suspension of ox erythrocytes was added to each tube. The tubes were transferred to 4° and the hemagglutination patterns were read in 3 hours. The highest dilution showing complete inhibition of hemagglutination was taken as the end-point.

Sera. Human, fetal calf, and calf sera were obtained from Microbiological Associates, Inc. Rabbit, mouse, and rat sera were obtained from healthy adult animals maintained at The Rockefeller Institute. Normal horse serum was obtained from the Department of Health of The City of New York.

Ovomucin. Ovomucin was prepared by the procedure of Gottschalk and Lind (1949), as modified by Hartley et al. (1959). The stock suspension contained a very fine granular precipitate which was always resuspended before use. The protein concentration, as measured by the procedure of Lowry (1951), was 10.3 mgm/ml. Ovomucin stored at 4° C did not lose inhibitory activity against the Dearing strain variant over a 9-month period.

Virus Structure

The size and some of the features of the fine structure of reovirus type 3 were determined by examination of negatively stained preparations (Brenner and Horne, 1959) in a Siemens Elmiskop I. Partially purified virus suspensions were obtained from lysates of infected cells and examined by Dr. Samuel Dales. The cell-associated virus was released by three cycles of freezing and thawing and the larger debris removed by centrifugation at 8700 g for 10 minutes. The clarified supernatants were then spun at either 78,400 or 92,700 g for 2 hours. The resulting pellets containing virus were treated in two ways. They were resuspended in PBS and examined within one hour to determine the virus diameter. Alternatively, the pellets were resuspended in 0.25 M ammonium acetate, pH 6.85, and were stored at -28° until examined. The original Dearing virus, but not the variant, required further purification. The pellet was brought to 3.0 ml

with reinforced Eagle's medium. 2.5 ml of Genetron 113 was added and the suspension was homogenized in a VirTis homogenizer at 4° C for 5 minutes. The mixture was centrifuged at 350 g at 4° C for 10 minutes. The aqueous supernatant was brought to 10 ml with PBS containing 5% fetal calf serum, centrifuged at 92,700 g for 2 hours, and the sediment was resuspended in 0.1 ml ammonium acetate.

The particle diameters of the original Dearing virus and the variant strain ranged from 620 to 840 A. The mean diameter of the original virus was 760 A and that of the variant 730 A. This difference is well within the experimental error. When the viruses were suspended in ammonium acetate instead of PBS, their diameters were approximately 20% less. A mean diameter of 560 A for the Dearing variant strain was obtained from suspensions in ammonium acetate. Rhim et al. (1961) measured the particle size of reovirus type 1, strain 716, stained by the negative contrast technique. Information on the suspending medium used is not available. The particles were reported to be approximately 750 A in diameter.

The best details of fine structure were obtained using preparations of reovirus 3 dried from ammonium acetate. Figure 1, A shows a low-power view of a typical preparation of the variant strain of Dearing virus. The morphological characteristics of the original Dearing strain (Figure 1, B) and the variant (Figure 1, C, D, and E) are closely similar. The virus surface or capsid is composed of either 5 or 6 sided prismatic capsomeres. Each capsomere is about 75 A wide, and possesses a hollow center 30-40 A in width and "walls" 15-20 A thick. A small proportion of structures which could be incomplete or damaged virus particles is present in these partially purified preparations (Figure 1, A, F). Also, scattered among virus particles are discrete units, often in clumps, of a similar size and morphology to that of the capsomeres (Figure 1, G). These could represent separated capsomeres of reovirus. In some fields very long filamentous structures having the width of virus particles were occasionally observed in these preparations. It is not certain, however, whether capsomeres are present on the surface of these filaments and therefore their relationship to the virus particles is not clear.

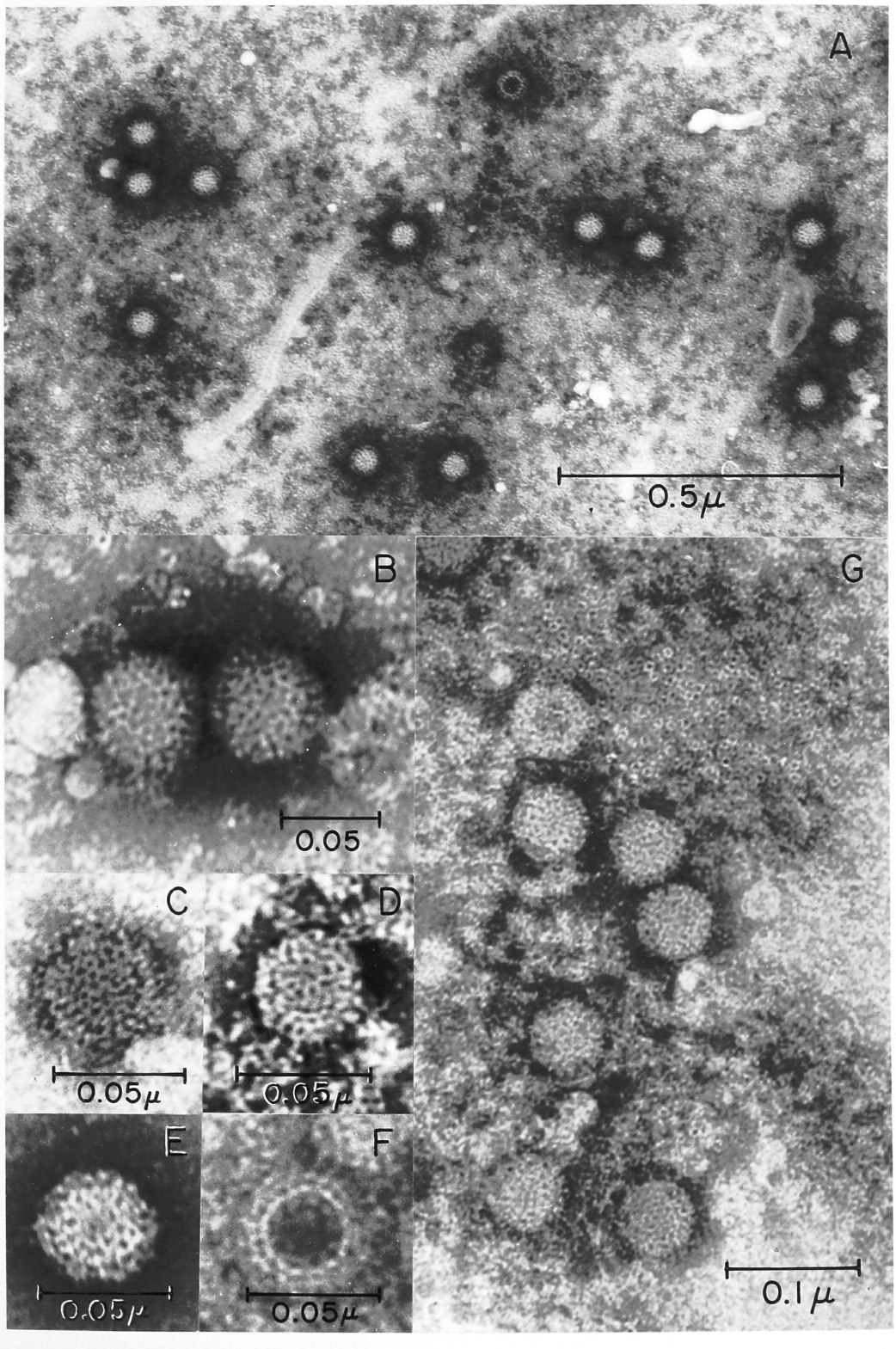


Figure 1. Electronmicrographs of reovirus, Dearing strain (B) and Dearing strain variant (A, C-G) (See text).

Hemagglutinating activity

Table I shows that reovirus type 3, original Dearing strain and its variant both agglutinated ox erythrocytes irrespective of whether the viruses were grown in monkey kidney or L cells. These viruses, grown in monkey kidney cells, also agglutinated sheep and human O cells, but the titers were lower; viruses grown in L cells failed to agglutinate sheep and human O cells. The tube agglutination patterns observed differed from those commonly seen with influenza virus in that cup-shaped sheets of agglutinated cells were not observed. The border of the agglutination patterns was irregular suggesting that the force of gravity had caused a partial collapse of the sheet. The Dearing strain and its variant did not agglutinate chicken, guinea pig, goose, nor rabbit erythrocytes.

Comparison of hemagglutination and infectivity titers

The hemagglutination activities and infectivities of several stocks of the Dearing strain variant were determined. The geometric means of the titers showed that 1 HAU (ox erythrocytes) of virus corresponds to 6.2×10^6 PFU (L cells) (cf. Table II).

Agglutination of bovine and human erythrocytes by reovirus type 3 strains

In collaboration with Dr. Hans J. Eggers, it was shown that the ability to agglutinate bovine erythrocytes was a general characteristic of reovirus type 3 and that reovirus types 1 and 2 lacked this characteristic. Table III shows the results obtained with eight reovirus type 3 strains; they all agglutinated stabilized bovine erythrocytes. Those strains which had a relatively low infectivity titer also gave relatively low hemagglutination titers; higher infectivity titers were correlated with higher hemagglutination titers. One additional virus preparation, designated Calf 814 strain, contained only $10^{5.4}$ $TCID_{50}$/ml and failed to agglutinate bovine erythrocytes, probably because of too low virus content. It should be stressed that with reovirus type 3, Dearing strain variant, 1 HAU (bovine erythrocytes) corresponded to 6.2×10^6 PFU (L cells).

Of the eight reovirus type 3 strains which agglutinated bovine erythrocytes, only four agglutinated human erythrocytes, and with these, the titers were lower with human than with bovine erythrocytes. Furthermore, the Nelson strain which had an infectivity titer of $10^{8.8}$ $TCID_{50}$/ml failed to agglutinate human erythrocytes, whereas the Calf 376H strain, with an

Table I

Agglutination of Erythrocytes from Different Species by Reovirus Type 3

Erythrocytes	Reovirus 3			
	Dearing, grown in monkey kidney cells	L cells	Dearing variant, grown in monkey kidney cells	L cells
Ox	64	32	64	64
Sheep	16	< 4	8	< 4
Human	4	< 4	4	< 4

Table II

Comparison of Hemagglutinating Activity and Infectivity of Reovirus 3, Dearing Strain Variant

Virus seed	Hemagglutinating activity[a] (HAU)	Infectivity PFU x 10^8	PFU/HAU x 10^6
A	64	3.7	5.8
B	128	6.0	4.7
C	128	11.9	9.2
Geometric mean	102	6.4	6.2

[a] Ox erythrocytes.

Table III

Hemagglutination of Bovine and Human RBC
by Reovirus Type 3 Strains

Strain	Source	Hemagglutination titer		Infectivity titer, log $TCID_{50}$/ml
		Bovine RBC (stabilized)	Human RBC (fresh)	
Calf 151J	Calf	16	< 4	6.5
Jones[a]	Man	16	< 4	not done
Dearing	Man	64	4	not done
Calf 808	Calf	64	< 4	7.5
Calf 376H	Calf	64	32	8.0
Nelson	Mouse	128	< 4	8.8
BVA6	Man	128	8	not done
Abney	Man	128	8	not done

[a] This strain is unrelated to the reovirus prototype 2 strain D5 (Jones)

infectivity titer of $10^{8.0}$, agglutinated human erythrocytes to a titer of 32. Thus, the ability of reovirus type 3 strains to agglutinate human erythrocytes appears to be an inconstant property of these viruses; in contrast, ability to agglutinate bovine erythrocytes is a constant property.

It should be emphasized that strain Jones of reovirus type 3 is unrelated to the reovirus type 2 prototype strain D5 (Jones).

Agglutination of bovine and human erythrocytes by reovirus types 1 and 2

As can be seen in Table IV, none of the 23 strains of reovirus types 1 and 2 agglutinated bovine erythrocytes although adequate amounts of each virus were present to agglutinate human erythrocytes.

Comparison of hemagglutination patterns and titers obtained with stabilized and freshly drawn bovine erythrocytes

Because the hemagglutination patterns produced by reovirus 3 with stabilized bovine erythrocytes rarely consisted of cup-shaped sheets of agglutinated cells, the patterns and titers obtained with stabilized suspensions and fresh erythrocytes were compared. It is shown in Table V that when freshly drawn cells were used, the hemagglutination titers were 2- to 16-fold higher than when cells from a stabilized suspension were used. Stable, cup-shaped sheets of agglutinated erythrocytes regularly formed when the freshly drawn cells were used.

Sensitivity to heat

Sterile tubes were preheated in water bath in which the temperature was maintained within 0.1° C of the desired value. Undiluted virus in Eagle's MEM supplemented with 5% fetal calf serum was added to the preheated tubes and at intervals, samples were removed, frozen rapidly in a dry ice-alcohol bath, and stored at -28° C. The samples were assayed one week later.

At each temperature, inactivation of the variant strain of Dearing virus was exponential (Figure 2). The half-life at 37°, 45°, and 56° was 157, 33, and 1.6 minutes respectively. The inactivation energy was computed to be 48,000 cal. per mole. It was reported recently that the half-life at 37° of reovirus type 1, strain 716, was 19 hours (Rhim et al., 1961). The virus studied in the present investigation was considerably more thermolabile.

Table IV

Hemagglutination of Bovine and Human RBC
by Reovirus Type 1 and 2 Strains

Type	Strain	Source	Hemagglutination titer	
			Bovine RBC (stabilized)	Human RBC (fresh)
I	Cocrel	Man	< 4	64
	Toluca no. 2182	Man	< 4	64
	CHHE 276	Man	< 4	128
	King	Man	< 4	128
	Calf 806	Calf	< 4	128
	Lang	Man	< 4	256
	Sampson, A.	Man	< 4	256
	Calf 104	Calf	< 4	256
	Calf 810	Calf	< 4	256
	Calf 802	Calf	< 4	512
	Calf 107	Calf	< 4	512
	CHHE 127	Man	< 4	1024
II	CHHE 318	Man	< 4	8
	D5 (Jones)	Man	< 4	64
	Tahiti no. 5283	Man	< 4	64
	Calf 237G	Calf	< 4	128
	Chimprhinitis 54	Chimpanzee	< 4	128
	Baden, S.	Man	< 4	128
	Calf 254A	Calf	< 4	256
	Amy	Man	< 4	256
	988	Man	< 4	512
	Gaither	Man	< 4	512
	Toluca no. 2442	Man	< 4	2048

Table V

Hemagglutination of Fresh or Stabilized Bovine RBC by Reovirus Type 3 Strains

Strain	Hemagglutination titer		Ratio of titers, fresh/stabilized bovine RBC
	Fresh RBC	Stabilized RBC	
Calf 151J	32	16	2
Calf 808	32	16	2
Dearing	256	64	4
Jones	128	16	8
Nelson	64	8	8
Calf 376H	256	16	16

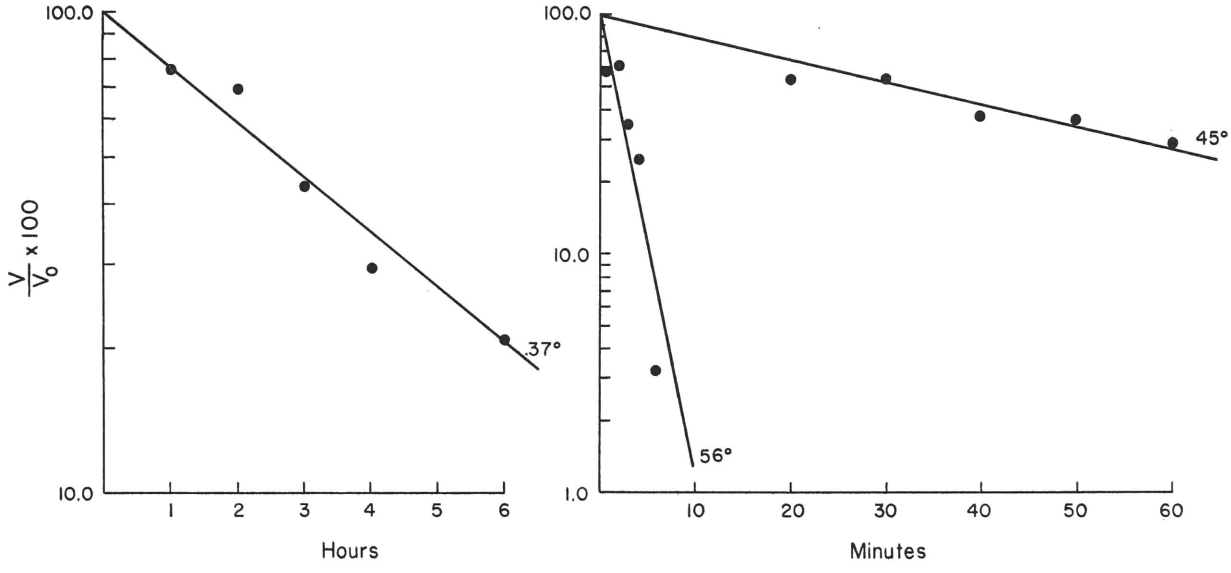


Figure 2. Heat inactivation of reovirus type 3, Dearing strain variant.

Sensitivity to Ether

Undiluted virus seed was exposed to 20% ethyl ether overnight at 4° C and the ether was then allowed to evaporate. A control sample, unexposed to ether, was handled similarly. The ether-treated and control samples were then assayed. As can be seen in Table VI, in two experiments, ether had no significant effect on the infectivity of the variant strain of Dearing virus. In another experiment, 50% inactivation was observed. These results are in agreement with previous reports that reoviruses are resistant to ether (Sabin, 1959; Rosen et al., 1960).

Inactivation of hemagglutinating activity and infectivity by trypsin and chymotrypsin

Trypsin was dissolved in phosphate buffer deficient in Ca^{++} and Mg^{++} (PD) (Dulbecco and Vogt, 1954) in a concentration of 1 mgm/ml. 0.5 ml of aliquots of Dearing variant virus stock in reinforced Eagle's MEM supplemented with 5% fetal calf serum were incubated at 37° for 1, 3, or 6 hours with an equal volume of trypsin solution. Controls consisted of virus stock diluted with equal volume of PD. At stated times, samples were placed in an ice bath and titered immediately.

As can be seen in Table VII, trypsin treatment for 1, 3, and 6 hours lowered the hemagglutinating activity to 50, 25, and $< 6.25\%$, and the infectivity to 32, 2.8, and 0.57% of the controls, respectively.

Treatment for 6 hours of both the Dearing and the Dearing variant strains with chymotrypsin under similar conditions left both the hemagglutinating activity and infectivity intact.

Inactivation of hemagglutinating activity and infectivity by PCMB

To virus stock which had been dialyzed for 9 hours against tris-buffered saline (TBS) was added an equal volume of PCMB solution giving a final concentration of sulfhydryl reagent of 10^{-4} M (Choppin and Philipson, 1961). A control preparation was diluted with an equal volume of TBS. The mixtures were incubated at 37°, and at stated times, samples were removed and placed in an ice bath until all samples were collected. The hemagglutination and infectivity titers were then determined. Undialyzed virus preparations were also used, but the effect of PCMB on such preparations was variable.

Table VI

Effect of Ether on the Infectivity
of Reovirus Type 3, Dearing Strain Variant

Experiment	PFU/ml x 10^7		
	Control	Ether-treated	Treated, % of control
A	31	25	81
B	11	5	45
C	15	13	87

Table VII

Inactivation of Hemagglutinating Activity and Infectivity of Reovirus 3, Dearing Strain Variant by Trypsin

Treatment, hours	Hemagglutination titer[a]		Infectivity, PFU/ml x 10^5	
	Control	Trypsin[b]	Control	Trypsin[b]
1	32	16	1400	450
3	32	4	1600	45
6	64	< 4	1400	8

[a] Ox erythrocytes
[b] 0.5 mg/ml

Table VIII shows that within 5 minutes after exposure to 10^{-4} M PCMB, the hemagglutination titer of reovirus 3, Dearing strain variant was reduced to 12.5%, and the infectivity titer to 0.78% of control. Further incubation did not affect the hemagglutination or infectivity titers. Attempts to reactivate samples of treated virus by incubation at room temperature for 30 minutes with an equal volume of glutathione in a final concentration of 5×10^{-3} resulted in no reactivation of either the hemagglutinating activity or infectivity.

It has been reported that potato virus X disintegrated into its protein subunits when exposed to excess PCMB or silver nitrate in tris buffer (Reichmann and Hatt, 1961). A pellet of partially purified Dearing virus variant, prepared by several cycles of differential centrifugation, was suspended in 10^{-2} M PCMB and incubated for 5 minutes at 37° and then kept in an ice bath until examined a few minutes later. PCMB reduced the hemagglutinating activity from 10,240 to 40 HAU, and the infectivity from 3.5×10^9 to less than 1.7×10^5 PFU. The PCMB-treated virus and control preparations were examined by Dr. Samuel Dales by the negative staining technique (Brenner and Horne, 1959). The particles in the two preparations were indistinguishable.

Sensitivity of ox and sheep erythrocyte receptors to V. cholerae filtrate

Serial two-fold dilutions of V. cholerae filtrate were made in 0.3 ml volumes in CaBBS, and to each was added 0.6 ml of 0.25% ox or sheep erythrocytes in 0.85% NaCl. The tubes were incubated for 1 hour at 37°, and then brought to room temperature. The erythrocytes were resuspended, and 4 HAU of reovirus 3, Dearing strain variant in 0.3 ml was added to each tube. The tubes were held at 4° for 3 hours and the hemagglutination patterns read.

V. cholerae filtrate, diluted 1:64, rendered ox and sheep erythrocytes inagglutinable by the variant as well as the original Dearing virus. With ox erythrocytes, a purified preparation of neuraminidase was also used, and similar results were obtained.

Susceptibility of reovirus 3 receptor on human erythrocytes to V. cholerae extract

Because the receptors on bovine erythrocytes for reovirus 3 are destroyed by V. cholerae filtrate, and because the receptors on human

Table VIII

Inactivation of Hemagglutinating Activity and Infectivity of Reovirus 3, Dearing Strain Variant by p-Chloromercuribenzoate (PCMB)

Treatment, minutes	Hemagglutination titer[a]		Infectivity, PFU/ml x 10^5	
	Control	PCMB	Control	PCMB
5	64	8	270	2.7
30	64	8	390	3.0

[a] Ox erythrocytes

erythrocytes for all types of reovirus are reportedly resistant to the action of RDE (Sabin, 1959), the interaction of several strains of reovirus 3 with human erythrocytes was investigated. Treatment of human erythrocytes for 1 hour at 37° with a V. cholerae filtrate had no effect on the weak agglutination of human erythrocytes by Dearing, Dearing variant, BVA6, and Abney strains, or on the stronger agglutination by calf 376H strain. Such treatment rendered bovine erythrocytes inagglutinable by reovirus 3.

Inhibition of hemagglutination by ovomucin

As seen in Table IX, an ovomucin solution with a protein content of 10.3 mgm/ml inhibited hemagglutination by the Dearing strain variant to a titer of 128. In contrast, ovomucin had no inhibiting effect on agglutination of human O cells by reovirus 2, Amy strain.

The ability of V. cholerae filtrate and of Lee virus to inactivate the hemagglutination inhibiting activity of ovomucin was determined. One ml of undiluted ovomucin was incubated for 12 hours at 37° with an equal volume of undiluted V. cholerae filtrate or BS. The mixtures were then heated at 56° for 30 minutes. As seen in Table IX, treatment with the V. cholerae filtrate reduced the hemagglutination inhibition titer of ovomucin from 128 to less than 8.

One ml of the ovomucin preparation was incubated for 12 hours at 37° with an equal volume of Lee virus suspension containing 20,480 HAU, or with BS, after which the mixtures were heated at 65° for 30 minutes. As can be seen in Table IX, treatment with Lee virus eliminated the inhibitory activity of ovomucin.

Attempts to destroy the hemagglutination inhibiting activity of ovomucin by treatment with the Dearing strain variant failed. In these experiments 2560 HAU of virus and 10.3 mgm of ovomucin were used and the virus-ovomucin mixture was incubated at 37° for 13 hours.

Inhibition of hemagglutination by normal sera

As can be seen in Table X, mouse, rabbit, rat, and calf sera, listed in order of decreasing activity, inhibited hemagglutination of ox erythrocytes by the Dearing strain variant. Fetal calf serum showed no inhibitory activity.

Treatment of mouse, rabbit, and rat sera with V. cholerae filtrate

Table IX

Inhibition of Hemagglutinating Activity of Reovirus 3, Dearing Strain Variant by Ovomucin

Treatment of ovomucin	Hemagglutination inhibition titer[c]
None	128
56°, 30 min.	128
V. cholerae filtrate[a], 37°, 12 hrs; 56°, 30 min.	< 8
65°, 30 min.	64
Lee virus[b], 37°, 12 hrs.; 65°, 30 min.	< 8

[a] 1:2 dilution.
[b] 20,480 HAU.
[c] 4 HAU of virus per tube; ox erythrocytes.

Table X

Inhibition of Hemagglutinating Activity of Reovirus 3, Dearing Strain Variant by Normal Sera

Species[a]	Number of sera in pool	Hemagglutination inhibition titer[b]
Mouse	20	64
Rabbit	1	32
Rat	4	16
Calf	Not known	8
Horse	1	8
Fetal calf	Not known	< 8

[a] Mouse, rabbit, and rat sera were heated at 56° for 30 min.; calf, horse, and fetal calf sera were not heated.

[b] 4 HAU of virus per tube; ox erythrocytes.

reduced markedly or eliminated their hemagglutination inhibiting activity for the Dearing strain variant. The inhibitory activity of normal calf, horse, and mouse sera on agglutination of human O cells by reovirus 2 was not affected by V. cholerae filtrate.

Discussion

Reoviruses are of medium size: the diameter of virus particles is 700-750 A. The viral protein coat, which contains no peripheral structural lipid, is made up of prismatic structural subunits. Two groups of workers (Vasquez and Tournier, 1962; Mayor and Melnick, 1962) have reported that reovirus particles possess cubic symmetry and that the number of structural subunits in reoviruses is 92.

Crick and Watson, 1956, proposed that the virus protein coat was probably made up of identical subunits and that in small spherical viruses these identical subunits were arranged in accordance with cubic symmetry and that the total number of subunits would be a multiple of 60. Considerable evidence has accumulated in support of Crick's and Watson's proposals (Horne and Wildy, 1961). Since there appear to be twelve 5-sided and eighty 6-sided structural subunits comprising the reovirus protein coat, the total number of sides is 540, a number divisible by sixty into an integral number and thus in accordance with the postulate.

Reovirus 3 appears to be the only reovirus type which agglutinates ox blood cells. Furthermore, the mechanism of reovirus 3 interaction with receptors on ox erythrocytes appears to be different from that involved in the interaction of reovirus 1 and 2 with human erythrocytes (Dardanoni and Zaffiro, 1958). Whereas V. cholerae filtrate renders ox erythrocytes inagglutinable by reovirus 3, such treatment has no effect on agglutination of human erythrocytes by reovirus 2. This finding suggested that reovirus 3 may be capable of reacting with neuraminic acid-containing receptors. Additional evidence to support this view was obtained in experiments with ovomucin and normal sera. Agglutination of ox erythrocytes by reovirus 3 was inhibited by ovomucin and normal mouse, rabbit, and rat sera, and in all instances, V. cholerae filtrate destroyed the inhibitory activity.

The ability of ovomucin to inhibit reovirus 3 hemagglutination was also destroyed by the Lee strain of influenza B virus. It should be emphasized that ovomucin did not inhibit hemagglutination of human O cells by reovirus 2, and that the hemagglutination inhibiting activity of normal calf, horse, and mouse sera against reovirus 2 was not affected by V. cholerae filtrate. It appears that neuraminic acid-containing receptors do not play an important role in the weak agglutination of human erythrocytes by reovirus 3.

Inactivation of the hemagglutinating activity as well as the infectivity of reovirus 3 by trypsin indicates that a protein component of reovirus surface may be involved in the interaction of this virus with cells. The fact that the hemagglutinating activity and infectivity of reovirus 3 could also be inactivated by p-chloromercuribenzoate (PCMB) has provided suggestive evidence that sulfhydryl groups of the virus protein may be essential for these activities. As PCMB also inactivates the hemagglutinating activity and infectivity of reovirus types 1 and 2 (Buckland, 1961; Allison, Buckland, and Andrewes, 1962) it appears that, certain differences in the mechanism of interaction not withstanding, a capsid with intact sulfhydryl groups or disulfide linkages is necessary for interaction of all three types of reovirus with erythrocytes.

It is known that PCMB and other sulfhydryl reagents are capable of inactivating both the infectivity and hemagglutinating activity of enteroviruses, whereas they inactivate the infectivity but not the hemagglutinating activity of influenza virus (Philipson and Choppin, 1960; Choppin and Philipson, 1961). It is of interest that PCMB, used at a concentration of 5×10^{-4} M, had no effect on the hemagglutinating activity or the infectivity of the LID-1 strain of polyoma virus. Table XI summarizes the information concerning the reaction of viruses with neuraminic acid receptors, and inactivation of viral infectivity and hemagglutinating activity by PCMB.

Thus, as far as the hemagglutinating activity is concerned, influenza and polyoma viruses, which react with neuraminic acid-containing receptors, are insensitive to the inactivating effects of PCMB, whereas enteroviruses, which do not react with such receptors, are inactivated by PCMB.

Clearly, reovirus type 3 belongs to a separate category in that

Table XI

Distinguishing Features of Virus-Receptor Interaction

	Reo			Entero	Myxo	Polyoma
	1	2	3			
Reaction of virus with neuraminic acid receptors	-	-	+	-	+	+
Inactivation of virus by PCMB						
Infectivity	+	+	+	+	+	-
Hemagglutinating activity	+	+	+	+	-	-

although it reacts with neuraminic acid-containing receptors, its hemagglutinating activity is inactivated by PCMB. A further distinction between reovirus 3 and influenza virus is that it, like polyoma virus (Hartley et al., 1959), appears to be unable to destroy neuraminic-acid receptors.

III. RESULTS, cont.

B. Dynamic Aspects of Growth of Reovirus 3 in L Cells.

Dynamic Aspects of Growth of Reovirus 3 in L Cells

It was early recognized that the cytopathic effect of reovirus in various cell lines was distinct from that of any virus that had been studied previously. There was a very long period after infection during which no changes were visible in the inoculated cells until an inclusion body formed usually in a perinuclear location in cells grown on monolayers (Malherbe and Harwin, 1957; Drouhet, 1960; Tournier and Plissier, 1960). The latter authors showed that the virus was present in infected cells only in the inclusion body; that there were no changes in the nucleus until very late in the infectious cycle at which time most of the virus had been made, and that the mitochondria remained intact throughout the viral growth cycle. When the inclusion body was examined histochemically (Drouhet, 1960), it was found to contain no Feulgen positive material, and no carbohydrate which could be detected with the periodic acid-Schiff reaction. The inclusion body failed to stain red with the methyl green-pyronin stain of Unna-Pappenheim after treatment with ribonuclease. It thus appeared that the virus probably contained RNA.

The findings of Tournier and Plissier (1960), who studied the intracellular development of reovirus type 3 by electron microscopy of thin sections, are of interest because they provide information as to the composition of the inclusions. Tournier and Plissier found that distributed around the nucleus there was a matrix containing granules of unequal size which were irregularly spaced. Only within this matrix were virus particles seen, either randomly distributed or in crystalline arrays. Also within the inclusions were filaments whose width was about 50% of the mean diameter of virus particles. In the present study, the filaments seen in suspensions of purified virus had the width of virus particles. The nature of the filaments seen within sections and those in suspension has remained unknown.

It was demonstrated that reovirus 3, Dearing strain, could infect L cells, and a plaque assay in L cells was described (Franklin, 1961). In the present section, a more detailed account of the growth of reovirus type 3 in L cells is given and evidence is presented that the genetic material of reovirus is indeed RNA.

Materials and Methods

Virus. Vaccinia virus seed was prepared by repeated passage in L cell monolayers of a stock which had been originally obtained from Dr. I. Hanafusa, Osaka University, Japan. The seed of vaccinia virus contained 3.3×10^6 PFU/ml.

Inhibitors. 5-Bromo-2'deoxyuridine was obtained from Cyclo Chemical Corp., Los Angeles, California. 5-Fluoro-2'-deoxyuridine was made available by Dr. R. Duschinsky of Hoffmann-La Roche Inc., Nutley, N.J. 4-Aminopteroylglutamic acid (aminopterin) was obtained from Mann Research Laboratories, New York, N.Y. 5,6-Dibromo-1-β-D-ribofuranosylbenzimidazole and Actinomycin D were made available by Dr. K. Folkers of Merck, Sharp and Dohme Research Laboratories, Rahway, N.J. Mitomycin C was made available by Dr. E.L. Tatum from a sample originally obtained from Drs. S. Ebashi, S. Tsuda, and Y. Noguchi, Kyowa Hakko Kohyo Co., Ltd., Tokyo (Japan).

Adsorption to cells

Complete monolayers of L cells in petri dishes were incubated with 50-80 PFU of virus in Eagle's MEM and incubated at 37° C. At intervals, groups of 3 to 5 plates were collected, washed 4 times with Eagle's MEM, overlayed with agar and returned to the incubator. Plaque counts were performed in the manner described above. The number of plaques observed after an adsorption period of 4 hours was considered equivalent to 100% and the adsorption curves were constructed accordingly.

Figure 3 shows one adsorption curve obtained with the original Dearing virus and two curves obtained in separate experiments with the variant strain. As can be seen, the adsorption curve of the original strain falls in between the two curves obtained with the variant strain. Thus, there appears to be no significant difference in the rates of adsorption of these viruses to L cells. The amount of virus adsorbed in two hours was 63-86% of that adsorbed in 4 hours. Addition of fetal calf serum (5-20%) to the adsorption medium affected neither the rate of adsorption of the variant strain nor the final amount of virus adsorbed.

In an earlier study with the Dearing strain in L cells, it was reported that maximum amount of virus adsorbed in 30 minutes (Franklin, 1961). In these earlier experiments adsorption was followed for only

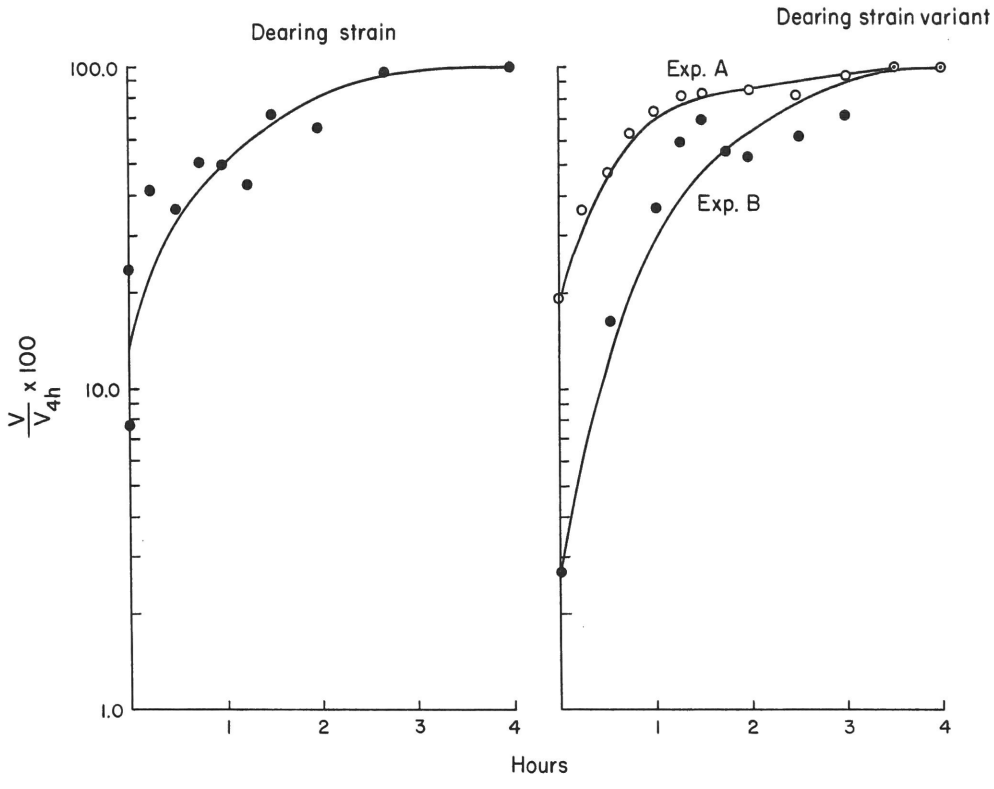


Figure 3. Adsorption of reovirus type 3, Dearing strain and Dearing strain variant to L cells.

1 hour. Rhim et al. (1961) followed the adsorption of reovirus type 1 to monkey kidney cells for 3 hours and found that approximately 90% adsorbed in 2 hours.

Growth curves

Incomplete monolayers were inoculated with the original Dearing virus and the variant strain at a multiplicity of 95:1. After a 2-hour period of adsorption at 37°, the cultures were washed 4 times, virus growth medium added (5 ml per plate) and the plates returned to the incubator. At intervals, the supernatant fluids from 3-4 plates were collected, pooled and centrifuged at 1200 rpm for 10 minutes. The supernatants which contained the released virus were stored at -28°. The sediments were added to cell samples which had been removed from the dishes with a rubber policeman. The samples were frozen and thawed 3 times and then centrifuged at 1200 rpm for 10 minutes. The cellular debris was discarded and the supernatants which contained cell-associated virus were stored at -28°.

As shown in Figure 4, the latent period for the Dearing virus was 7 hours. Cell-associated virus (CAV) increased exponentially from 7 to 11 hours and reached a maximum at 15 hours. Exponential increase in released virus (RV) occurred from 9 to 13 hours and RV reached a maximum at 15 hours. The latent period for the variant strain of Dearing virus was also 7 hours. With the variant, exponential increase in CAV continued until 13 hours and the CAV reached a maximum at 15 hours; increase in RV was nearly exponential from 11 to 17 hours.

Thus, the curves describing exponential increase in CAV and RV were separated by 3 hours for the Dearing virus, but by 6 hours for the variant strain. Furthermore, when the maximum yields were reached, 22% of the Dearing virus, but only 7% of the variant strain was released.

The final yield of the two viruses was similar, but it varied from 300 to 2600 PFU per cell in individual experiments. This variation was not related to the number of cells present in cultures.

In earlier work with reovirus type 1 (Rhim et al., 1961) in monkey kidney cells in which a multiplicity of 4.7 PFU per cell was used, the latent period was between 6 and 12 hours and maximum yields were reached

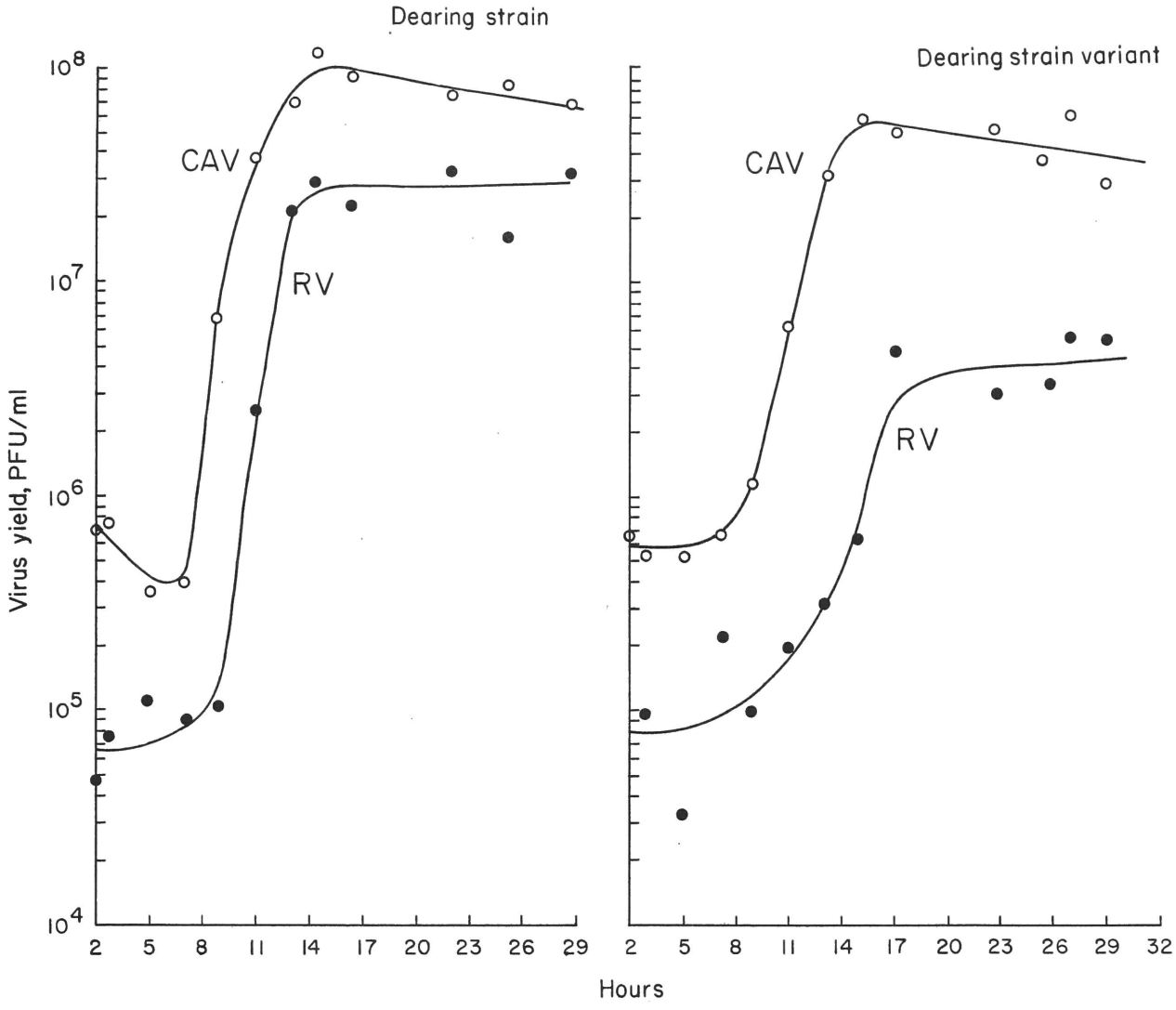


Figure 4. Growth curves of Dearing strain and Dearing strain variant in L cells. Cell associated (CAV) and released virus (RV) are plotted as a function of time in hours.

in 48-54 hours. In the system used by Rhim and coworkers, the average yield was 225 PFU per cell and extra-cellular virus constituted 15-25% of the total.

Cytological changes

The cytological changes in L cells infected with the Dearing virus or the variant strain were similar. Phase contrast microscopy showed that 14 to 15 hours after inoculation granular material began accumulating in the perinuclear area. Between 15 and 16 hours the nuclei in some cells became partially or completely surrounded by granular material. The number of cells showing such inclusions increased rapidly, and by the 17th hour the inclusions showed signs of breaking up in some cells. By this time a few cells had rounded up and come off of the petri dish.

Staining of cells with fluorescein-labeled antibody showed that viral antigen was localized within the inclusions, as was demonstrated earlier by Drouhet (1960), who studied reovirus type 1, Lang strain, in monkey kidney cells.

For cytochemical studies, cultures were inoculated as described above. At intervals after inoculation, groups of coverslips were removed, washed once in PBS, quenched in absolute ethanol-dry ice mixture for 10 seconds and substituted in absolute ethanol-methanol mixture (1:1) at -28^{o} for 60 minutes with a change of ethanol-methanol mixture every 15 minutes. The coverslips were then dried in a stream of cold air and stored at -28^{o}. The cells were brought to acetate buffer (0.1M, pH 5.6) through a series of alcohol washes containing increasing percentage of water, washed two times in acetate buffer for 5 minutes each, and stained for 15 minutes at room temperature with 0.05% acridine orange (Chroma-Gesellschaft, Stuttgart) in acetate buffer, pH 5.6. The coverslip cultures were then washed three times for 10 minutes each with buffer, mounted in acetate buffer containing 20% glycerin, sealed with Kronig's cement, and stored at 4^{o} C.

Figures 5 and 6 show control and inoculated L cell monolayer cultures stained with 0.05% acridine orange in acetate buffer at pH 5.6. The inclusions stained orthochromatically pale green. Direct application of DNase left the inclusions intact; nor were they removed by treatment with

Figure 5. Color photomicrograph of uninoculated L cell monolayer culture. The cells have been fixed and then stained with acridine orange. The preparation was examined and photographed with a Zeiss Ultraphot II photomicroscope using an Osram 200 watt high pressure mercury burner, a BG 12 exciter filter, and an OG 1 barrier filter. For photography Supernascochrome 120 daylight film was used. x560

Figure 6. Color photomicrograph of L cell monolayer culture infected at a high multiplicity with reovirus 22 hours previously, fixed, and then stained with acridine orange. Examination and photography as in Figure 5. x560

.002% pepsin in 10% acetic acid for 5 minutes at room temperature, followed by 0.01% DNase in .003 M $MgCl_2$ for 1 hour at 37°, and finally by a second pepsin treatment - a procedure known to remove viral DNA (Peters and Stoeckenius, 1954; Mayor, 1961). Both the Feulgen stain, and also the fluorescent Feulgen stain using the Acriflavine-Schiff reaction (Ornstein, 1957), gave negative results. Thus, the inclusions apparently did not contain DNA. The inclusions were resistant to 0.01% RNase in 0.003 M $MgCl_2$ for 1 hour at 37° with or without prior exposure for 5 minutes to 0.002% pepsin. Treatment with RNase with both pre-and post-treatment for 5 minutes with pepsin completely removed the inclusions, whereas such treatment in the absence of RNase removed only some of the inclusions. Thus, despite orthochromatic staining with acridine orange, the inclusions appear to contain RNA.

It should be emphasized that the perinuclear inclusions still stained orthochromatically green 22 hours after virus inoculation, i.e. 7 hours after maximum virus yields were reached. It should also be noted that in control experiments in which infected cells were incubated in 0.003 M $MgCl_2$ for 1 hour at 37° without DNase or RNase, $MgCl_2$ appeared to stabilize partially the inclusions against the effects of pre- and postreatment with either acid alone or pepsin in acid. When the cells were incubated in deionized water instead of $MgCl_2$, the inclusions were partially removed by pre- and posttreatment with 10% acetic acid, and completely removed by pre- and posttreatment with pepsin in acetic acid.

In a cytological study with acridine orange of monkey kidney cells infected with reovirus type 1, Rhim et al. (1962) noted that at about the time of maximal virus yield, there was present a brilliant red perinuclear inclusion, sensitive to ribonuclease. This ribonuclease-sensitive inclusion appeared to develop from within a pale-green inclusion which was present earlier and which was sensitive to pepsin but not the nucleases. One hundred-fold and five-fold higher concentrations of pepsin and ribonuclease, respectively, were used by Rhim and associates (1962) than in the present study.

Effect of inhibitors on virus multiplication

Metabolic inhibitors were used to obtain evidence as to the type of nucleic acid present in reovirus. Plastic dishes were seeded with

between 3 x 10^5 - 1 x 10^6 L cells. Eighteen to 24 hours later, the medium was replaced with growth medium containing one of several inhibitors, or with control medium. Two to four plates were used per variable. After 24 hours, medium was removed, dishes washed once, and 0.3 ml of virus seed without inhibitor inoculated. The virus/cell multiplicities were as follows: Dearing virus, 65-154 PFU/cell; Dearing virus variant, 56-155 PFU/cell; vaccinia virus, 0.9-1.7 PFU/cell. The cells from two to four dishes were counted after removal from the plates with 0.25% trypsin - 0.05% versene in phosphate buffer deficient in Ca^{++} and Mg^{++}. After a 2-hour adsorption period, the cultures were washed four times and growth medium with or without inhibitor was added. The cultures were collected 19 to 24 hours after virus inoculation, frozen and thawed three times, and then centrifuged at 1200 rpm for 10 minutes. The cellular debris was discarded and the supernatants were stored at -28° C. The yield of virus per cell was determined by the plaque technique and the yield in treated cultures was expressed as per cent of that in controls. The effects of 5-bromo-2'-deoxyuridine and 5,6-dibromo-1-β-D-ribofuranosylbenzimidazole on Dearing virus multiplication were studied in only a single experiment. Otherwise, 2-5 experiments were performed with each compound and virus, and the results were averaged. Only the mean percentage yields are shown in Tables XII and XIII whereas in Table XIV the results of a triplicate experiment are given in PFU/ml. The degree of variation in results in Table XIV is representative of that in most experiments performed.

As can be seen in Table XII, the inhibitors were used at widely different concentrations because of their differing activities. The yields of Dearing virus and of the variant strain were comparable, but in general the Dearing virus showed slightly greater sensitivity to inhibitors than the variant strain.

5-Fluoro-2'-deoxyuridine (FUDR) and 5-bromo-2'-deoxyuridine (BUDR) had no effect on the yield of reovirus, but they caused 90-99% inhibition of vaccinia virus. 5,6-Dibromo-1-β-D-ribofuranosylbenzimidazole markedly reduced the yields of both reo and vaccinia viruses. 4-Aminopteroylglutamic acid (aminopterin) also caused considerable inhibition of each of these viruses. In an experiment in which aminopterin, 10γ/ml, was added after virus adsorption, the yield of Dearing virus variant was 28% of control,

Table XII

Effect of Inhibitors on the Multiplication of Reo 3 and Vaccinia Viruses

Compound	Concentration, μg/ml	Yield of virus, % of control		
		Reo		Vaccinia
		Dearing	Dearing Variant	
5-Fluoro-2'-deoxyuridine (FUDR)	0.25	-	88	12
5-Bromo-2'-deoxyuridine (BUDR)	100	69	109	0.8
5,6-Dibromo-1-β-D-ribofuranosylbenzimidazole	39	3.9	10	1.6
4-Aminopteroylglutamic acid (Aminopterin)	10	9.1	25	9.7
Mitomycin C	1.0	-	87	9.9
	5.0	11	18	0.8

i.e. closely similar to that obtained when the compound was present both before and after virus inoculation. Mitomycin C had a much greater effect on the yield of vaccinia than reovirus. Indeed, at 1 μg/ml of Mitomycin C the yield of reovirus was not significantly depressed.

With Actinomycin D, the procedure of treatment was varied. As with other compounds, some cultures were treated for 24 hours before virus inoculation, and subsequently the inhibitor was present from the time of completion of adsorption to termination of the experiment. In other cultures, Actinomycin D was present only before or after inoculation. As can be seen in Table XIII, the original and variant strains of Dearing virus, and vaccinia virus were all inhibited to a similar extent by combined pre- and post-inoculation treatment. Likewise, pre-treatment alone inhibited the original Dearing strain and the vaccinia virus to a similar extent. However, post-inoculation treatment alone was more effective with vaccinia than with reovirus.

Further experiments were done with Actinomycin D in which the cells were exposed to the inhibitor for 8 hours, washed once, and incubated for 16 hours in growth medium in the absence of the inhibitor. The pretreated and control cells then received virus. Results of a triplicate experiment performed on the same day are shown in Table XIV. An 8-hour pulse of Actinomycin D, 2 μg/ml, reduced the yields of the original and variant strain of Dearing virus, and of vaccinia virus to 52, 46, and 37% of the untreated controls, respectively. After an 8-hour pulse of Actinomycin D at 5 μg/ml, the treated cells produced these viruses in amounts equal to 23, 15, and 16% of the yields in controls. The degree of inhibition of vaccinia virus by Actinomycin D observed in the present experiments was somewhat less than that found by Reich et al. (1962).

Thus, when cells are pretreated with Actinomycin D, their ability to support reo and vaccinia virus multiplication is impaired to a similar degree. It should be reemphasized that Actinomycin D, present during the post-inoculation period only, inhibits vaccinia virus considerably more than reovirus.

Discussion

A variant of reovirus type 3, Dearing strain, which emerged after

Table XIII

Inhibition of Reo and Vaccinia Virus Multiplication by Actinomycin D

Treatment Actinomycin D, 2 μg/ml		Yield of virus, % of control		
		Reo		Vaccinia
Pre[a]	Post[b]	Dearing	Dearing Variant	
+	+	0.4	0.9	0.7
+	0	7.0	-	10
0	+	6.0	11	0.3

[a] For 24 hours before virus inoculation.
[b] For 22 hours after virus inoculation.

Table XIV

Inhibition of Reo and Vaccinia Virus Multiplication by Actinomycin D Given in an 8-Hour Pulse

Treatment	Number of cells per plate in uninfected cultures, $x10^6$	Yield of Virus					
		Reo				Vaccinia	
		Dearing		Dearing Variant			
		PFU/cell	% of control	PFU/cell	% of control	PFU/cell	% of control
None	1.0	1800[a]	100	1300[b]	100	30[c]	100
Actinomycin, 2 μg/ml	0.33	740	41	580	45	8.4	28
		960	53	760	59	7.3	24
		1100	61	430	33	18	60
Actinomycin, 5 μg/ml	0.43	420	23	230	18	4.1	14
		540	30	100	7.7	2.9	9.7
		270	15	250	19	6.9	23

[a] Mean of 1800, 1800, and 1900 PFU/ml, representing 3 separate groups of cultures.
[b] Mean of 1600, 1200, and 1100 PFU/ml.
[c] Mean of 30, 30, and 29 PFU/ml.

repeated passage of the original virus in L cells, was released from infected cells to a lesser extent than the original virus which had been passaged in L cells only 4 times. The variant differed from the original virus also in being less sensitive to specific antibody. In antigenic constitution, however, it was indistinguishable from the original. It is of interest that a variant of ECHO 6 virus which emerged on repeated passage in monkey kidney cells was, in contrast to the present findings, more sensitive to antibody (Karzon et al., 1959). A further difference between the original Dearing virus and the variant strain is the slightly greater sensitivity of the original virus to metabolic inhibitors.

It should be emphasized that in several other respects the two strains were indistinguishable. Their particle diameters were not significantly different and both possessed five- or six-sided capsomeres with hollow centers. No differences were found in the dimensions of the capsomeres of the two viruses. The strains adsorbed to L cells at similar rates, showed latent periods of similar duration, reached maximal yields at similar times, and caused similar pathological changes in L cells. The observation that the latent period was 7 hours and that the maximal yields were reached at 15 hours is noteworthy. Isaacs (1959) has pointed out that, in general, RNA viruses have a shorter lag period and a faster rate of increase than DNA viruses. To support his contention, Isaacs (1959) cited 6 examples; to these, several may be added (Bachrach et al., 1957; Franklin and Henry, 1960; Eggers and Tamm, 1961; Franklin, 1962b; Ginsberg, 1958). If, as the evidence suggests, reovirus is an RNA virus, it is unusual in that its multiplication is relatively slow compared to that of many RNA viruses.

The earliest detectable cytological changes, consisting of accumulations of granular material in the perinuclear area, appeared at the time when maximal virus yields were reached. Soon afterwards, perinuclear inclusions developed which, in many cells, completely surrounded the nucleus. A short time after full development, the inclusions showed evidence of fragmentation and the cells began rounding. The results of the cytochemical studies indicated that the inclusions do not contain DNA.

Additional evidence that reovirus nucleic acid is not DNA was obtained in studies with metabolic inhibitors. The multiplication of

reovirus was not inhibited by FUDR or BUDR. The fluoro derivative is an inhibitor of thymidylate synthesis (Cohen et al., 1958; Rich et al., 1960), whereas the bromo derivative is known to be incorporated into DNA (Hakala, 1959). Both compounds cause marked inhibition of the DNA-containing vaccinia virus (Salzman, 1960; Simon, 1961). FUDR is also a highly active inhibitor of adenovirus (Flanagan and Ginsberg, 1961) and herpes virus multiplication (Newton and Tamm, 1959, unpublished results). On the other hand this compound does not inhibit the multiplication of polio (Salzman, 1960), influenza, and Newcastle disease viruses (Tamm and Newton, 1959, unpublished results), and BUDR failed to inhibit NDV and poliovirus (Simon, 1961). Thus, the available evidence, though somewhat limited, is fully in accord with the view that FUDR and BUDR inhibit DNA, but not RNA viruses.

The results of studies with aminopterin and riboside of 5,6-dibromobenzimidazole are not incompatible with the view that reovirus nucleic acid is RNA, but they do not provide a basis for specific conclusions concerning the nature of the viral nucleic acid. Aminopterin, an antagonist of folic acid, would be expected to interfere not only with DNA synthesis but also with protein and RNA metabolism, because folic acid serves many different functions (Holland, 1961). In previous investigations on virus inhibition, aminopterin was found to be markedly inhibitory for vaccinia virus, but had little or no effect on NDV and polioviruses (Simon, 1961). Inhibition of reovirus multiplication by aminopterin may indicate that this virus is quantitatively more fastidious in its metabolic requirements than NDV or poliovirus. 5,6-Dibromo-1-β-D-ribofuranosyl-benzimidazole would be expected to inhibit the multiplication of both RNA- and DNA-containing viruses (Tamm et al., 1956; Tamm et al., 1960); in the present experiments it inhibited both reo and vaccinia virus multiplication.

In experiments reported earlier, Mitomycin C and Actinomycin D, administered under restricted conditions with respect to time and duration of treatment, inhibited the multiplication of vaccinia virus (DNA), but not that of Mengo virus (RNA) (Reich and Franklin, 1961a; Reich et al., 1961b). In the present experiments, vaccinia virus was considerably more sensitive to inhibition by Mitomycin C and Actinomycin D than reovirus, except when cells were pre-treated with Actinomycin D followed by removal

of the inhibitor before virus inoculation. The ability of pre-treated cells to produce reo or vaccinia viruses was restricted to a similar degree. It is likely that under the conditions of pre-treatment, the metabolism of cells had been altered in several respects by the time virus was introduced, and, therefore, selective effects were obscured. It is indeed remarkable that Mengo virus multiplies to full yields under these conditions. This probably indicates that Mengo virus is less fastidious in its metabolic requirements than reo and vaccinia viruses. However, Mengo virus is also characterized by a rapid growth rate; it can be harvested in single cycle experiments in 10-12 hours, whereas approximately twice as long periods are required with reo and vaccinia viruses. This may also be a factor of importance in determining the outcome of inhibition experiments.

To summarize, the results of cytochemical and pertinent inhibition experiments indicate that the nucleic acid in reovirus type 3 is probably RNA.

III. RESULTS, cont.

C. Macromolecular Synthesis in Reovirus-Infected L Cells

Macromolecular Synthesis in Reovirus Infected L Cells

As described in the preceding section, the reproductive process of reovirus type 3 is characterized by three features which distinguish it from other RNA viruses. The multiplication of reovirus is relatively slow compared to many RNA viruses; the inclusion body which develops in reovirus-infected cells stains orthochromatically greenish-yellow with acridine orange, as if it contained DNA, yet it contains RNA; and finally in contrast to all other cytoplasmic RNA virus studied (Reich et al., 1962; Shatkin, 1962; Barry et al., 1962), the reproduction of reovirus is inhibited by Actinomycin D. The results of the present experiments have shown that in its effect on the synthesis of DNA, RNA, and proteins in infected cells, reovirus is also unusual.

Materials and Methods

The tritium-labeled compounds were obtained from New England Nuclear Corporation, Boston, Mass. The specific activities of thymidine-methyl-H^3, uridine-H^3, and DL-Leucine-H^3 were respectively 6.8, 6.8, and 4.2 mc/μmole.

Macromolecular syntheses were followed during the viral growth cycle by conventional procedures of autoradiography employing the film stripping technique. L cells grown on cover slips were infected with reovirus type 3 (Dearing strain) at a virus:cell multiplicity of 100:1, and exposed at various times to labeled precursors, i.e. tritiated thymidine, uridine, or leucine, for a pulse of 15 minutes.

Effect on DNA Synthesis

Figure 7 shows the effect of reovirus infection on cellular DNA synthesis. After the period of virus adsorption, an increase in the number of nuclear grains was observed in both the inoculated and control cultures. This increase to a steady state level can be attributed to recovery from the effects of the untoward conditions of the adsorption phase during which the monolayer was incubated for 2 hours in only 0.3 ml of medium. During other periods of incubation, the volume was 5 ml. Between 9 1/4 and 10 1/4 hours there became apparent a striking inhibition of thymidine incorporation into DNA in the inoculated cultures whereas the control cultures continued to incorporate thymidine at a steady rate. It is during this time that virus is increasing exponentially in the inoculated cultures. At the time

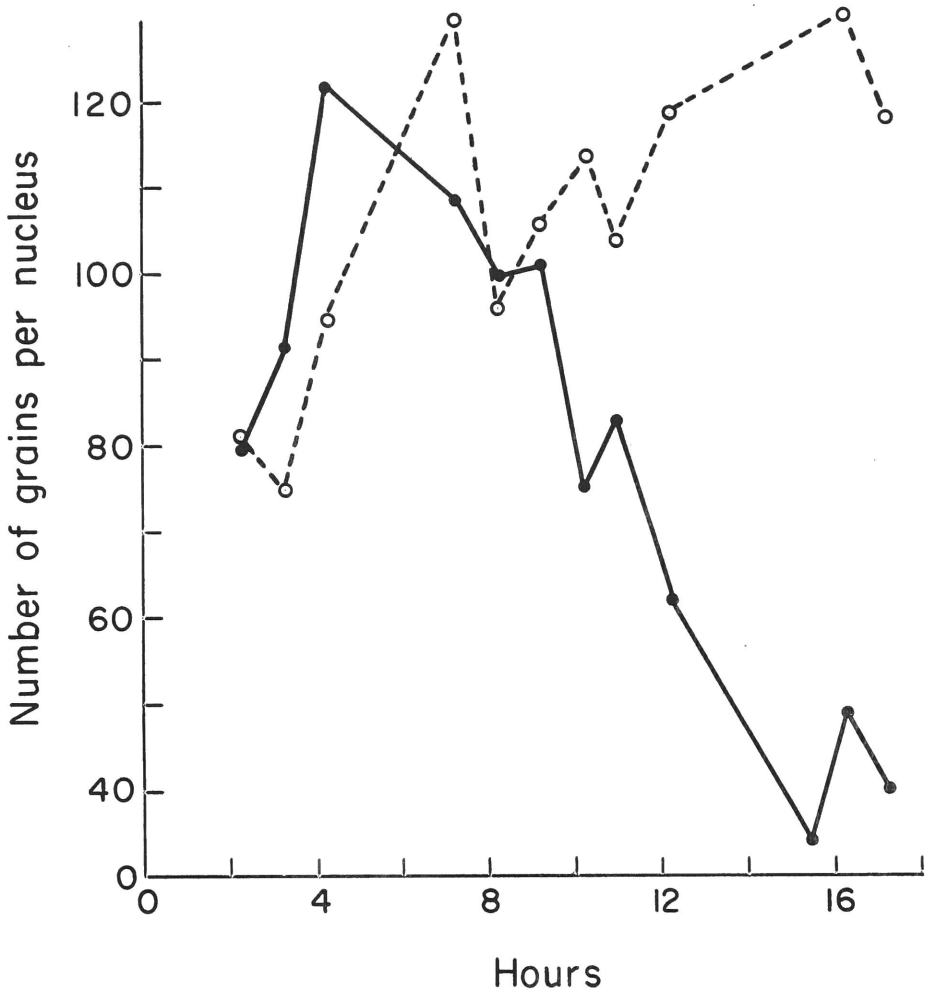


Figure 7. Effect of reovirus infection on the rate of thymidine incorporation into DNA of L cells. At various times growth medium was removed and the cells were exposed to thymidine-H^3, 0.25 μc/ml (specific activity 6.8 mc/μmole). The medium containing labeled thymidine was then removed, and the cells incubated for 15 minutes in the presence of 1000-fold excess of unlabeled thymidine. The cells were fixed in acetic acid-alcohol (1:3) for 10 minutes at 4°, and carried through a series of alcohol washes (90, 70, 40, 20, and 10%) into 5% perchloric acid. They were extracted for 1 hour at 4° and washed exhaustively with water, then alcohol, alcohol-ether 1:1, and ether, and dried. Kodak film AR-10 was used in the preparation of autoradiographs. The film was exposed for 4-7 days, developed, and stained with a modified Giemsa stain (Gude, Upton, and Odell, 1955). 25 cells were counted in the control and in the inoculated samples. .____. inoculated; o----o control.

that maximal yields of virus were reached, that is at 15 hours, DNA synthesis in infected cells had become inhibited to the extent of 80%. These results demonstrate clearly the effect of reovirus infection on the rate of DNA synthesis in those cells which were in the DNA-synthetic phase. To find out whether reovirus infection had an effect on the initiation of DNA synthesis, the proportion of cells with nuclear grains was determined by counting. As can be seen in Figure 8, there was as high a percentage of cells with nuclear grains in inoculated as in control cultures.

Effect on RNA Synthesis

Figure 9 shows the effect of virus infection on the incorporation of uridine into RNA. There was no inhibition of RNA synthesis evident during the entire growth cycle. Indeed, experiments in which RNA synthesis was followed until 5 hours after maximal yields of virus had been reached showed no inhibition of RNA synthesis although at this time some cells were coming off the glass. Figure 10 shows that reovirus infection had no effect on the rate of nucleolar RNA synthesis.

Effect on Protein Synthesis

Figure 11 shows the results of a representative experiment on incorporation of leucine into proteins. As can be seen, there was no evidence of inhibition of protein synthesis by reovirus infection.

Discussion

It thus appears that reovirus infection specifically inhibits the process of cellular DNA synthesis, although it does not inhibit the initiation of the DNA-synthetic phase. The inhibition of DNA synthesis occurs without any apparent effect on the two other principal areas of macromolecular synthesis. It should be emphasized, however, that with the techniques used, the macromolecules which are normal cellular components cannot be differentiated from those which may be viral or virus-induced. Thus, inhibition of cellular RNA or protein synthesis could be masked by virus-controlled syntheses. However, the close similarity in the overall rates of RNA and protein synthesis in infected and control cells suggests that reovirus did not have any major effects on the synthesis of these cellular components.

With three other cytoplasmic RNA viruses (Franklin and Baltimore,

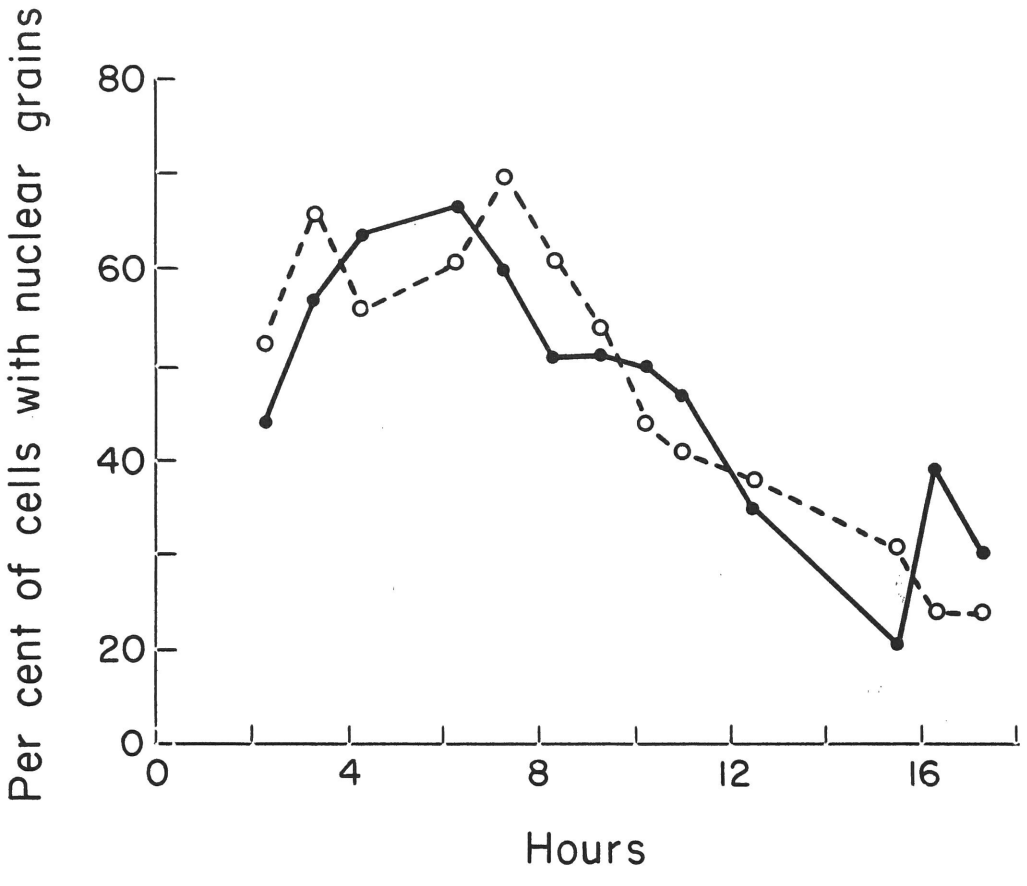
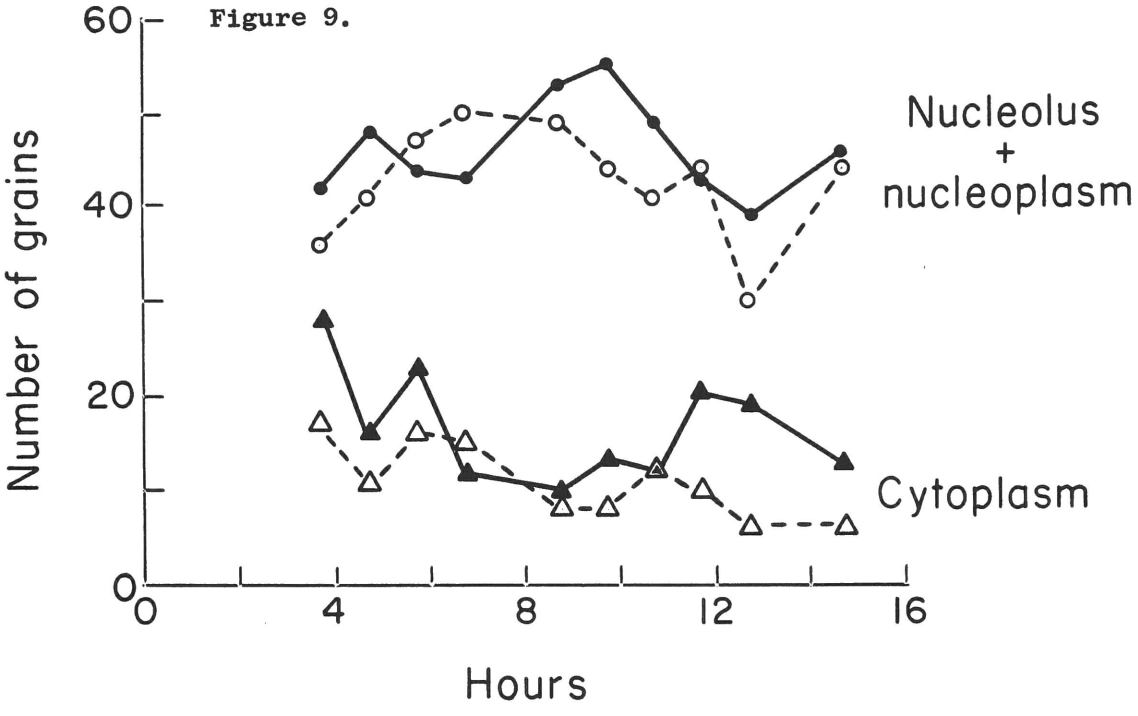
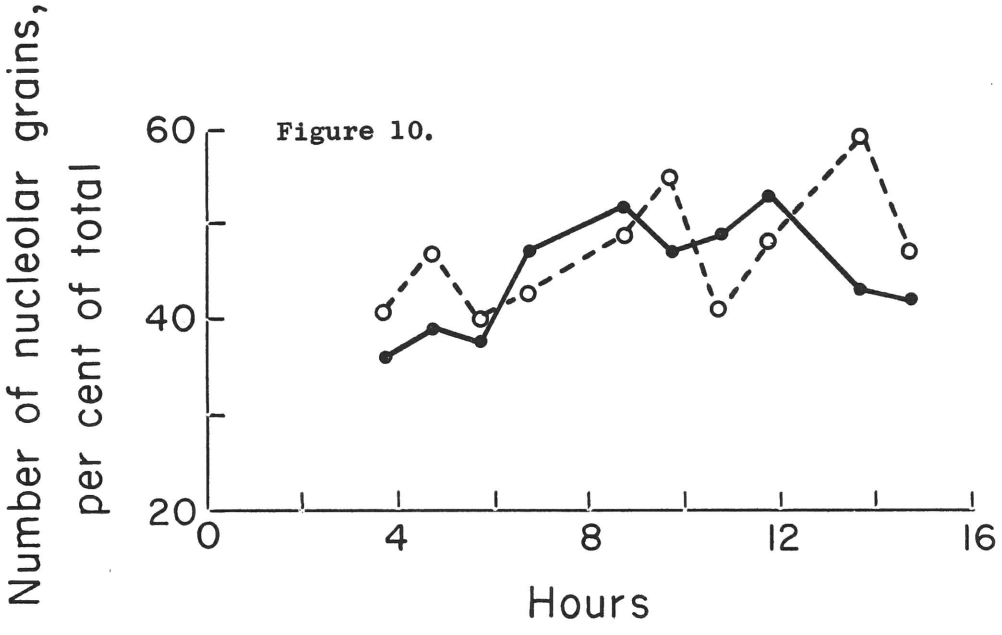


Figure 8. Effect of reovirus infection on the percent of cells in the DNA synthetic phase. The curves are based on data obtained from the experiment described in Figure 7.





Figures 9 and 10. Effect of reovirus infection on incorporation of uridine into RNA in L cells. Procedure as in Figure 7. The precursor used was uridine-H^3, 1 μc/ml (specific activity 6.8 mc/μmole), and the medium contained a 1000-fold excess of unlabeled thymidine. During the 15 minutes which followed exposure to uridine-H^3, the cells were incubated in growth medium containing a 1000-fold excess of unlabeled uridine and thymidine. ._____. inoculated; o----o control.

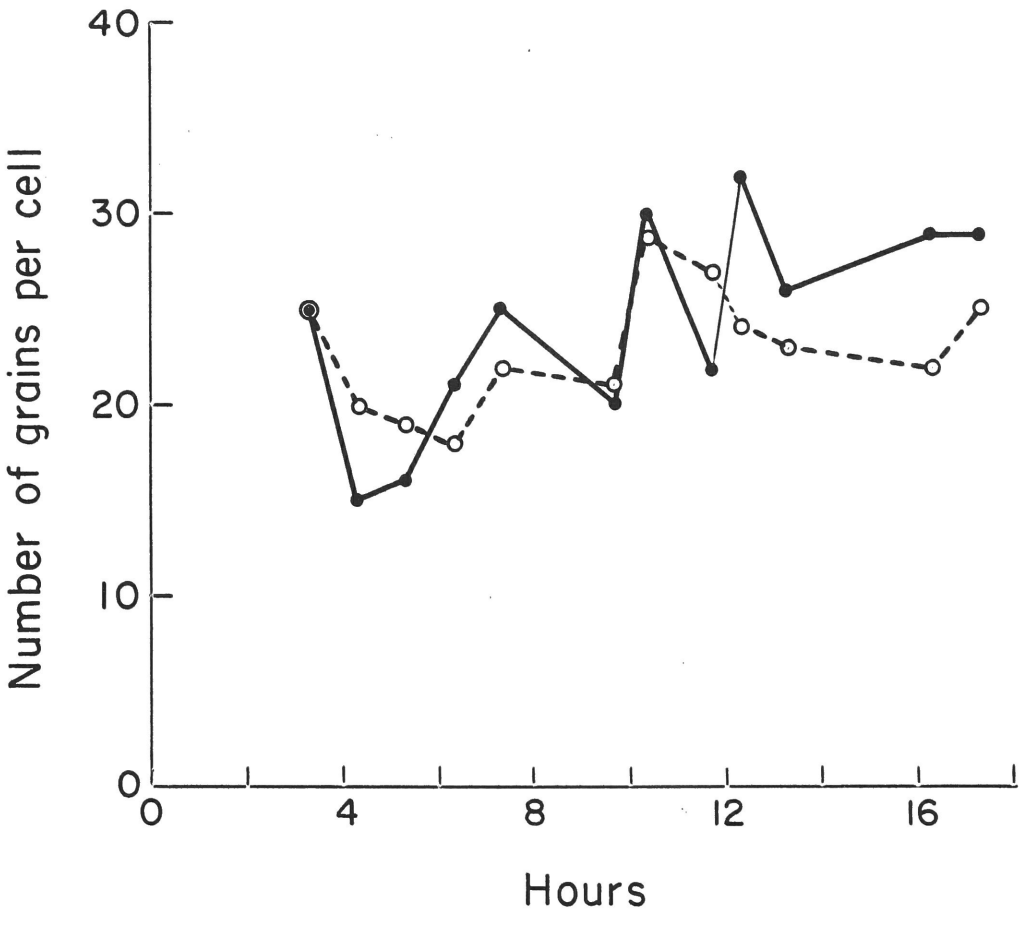


Figure 11. Effect of reovirus infection on incorporation of leucine into proteins in L cells. Procedure as in Figure 7. The precursor used was leucine-H^3, 1 μc/ml (specific activity 4.2 mc/μmole), and the medium lacked both serum and unlabeled leucine. After exposure to leucine-H^3, the cells were incubated for 15 minutes in growth medium. Perchloric acid extraction was done at 37° rather than at 4°. .____. inoculated; o----o control.

1962; Scholtissek et al., 1962; Kerr et al., 1962; Levy, 1961; Salzman et al., 1959; Wheelock and Tamm, 1961) and also with vaccinia virus (Kit and Dubbs, 1962), there has been noted an early and profound depression of cellular RNA or protein synthesis or both with a concomitant or subsequent inhibition of DNA synthesis. In these instances, it has not been possible to attribute the inhibition of DNA synthesis to a primary effect on the DNA-synthetic process itself, since either RNA or protein synthesis has also been depressed; in the case of reovirus, the inhibition of DNA synthesis after infection appears to be a primary and specific event.

III. RESULTS, cont.

D. Secondary Structure of Reovirus RNA

Secondary Structure of Reovirus RNA

The findings described provided a strong stimulus to undertake an investigation of the chemical structure of reovirus RNA, and to ascertain whether this component possessed distinguishing features which were responsible for the unusual behaviour of the virus. Of the several features which distinguish reovirus from other RNA viruses, the one which in fact suggested a specific structural feature of reovirus RNA was the anomalous staining with acridine orange. The question as to why the inclusions stained orthochromatically pale-green with acridine orange is an interesting one. It is possible that the inclusions contained RNA, which even after fixation combined only with a relatively small amount of acridine orange. Such behaviour would be unusual for RNA under the conditions of dye concentration and pH used (Schummelfeder, 1958; Mayor and Diwan, 1961). One possible explanation for low binding of dye which should be considered is that the RNA may be double-stranded. This is suggested by the evidence that double-stranded DNA stains orthochromatically, whereas single-stranded DNA shows metachromatic staining (Mayor and Hill, 1961; Bradley and Felsenfeld, 1959).

The experimental results about to be described provide strong evidence that the RNA of reovirus is in fact double-stranded. In these experiments only virus released from cells was used.

Materials and Methods

Ribonuclease, bovine pancreas, 5x crystallized, and desoxyribonuclease, beef pancreas, 1x crystallized, were obtained from Mann Research Laboratories, Inc., New York, New York.

Bovine albumin powder, fraction V from bovine plasma, was obtained from Armour Pharmaceutical Company, Kankakee, Illinois.

Diphenylamine, special indicator grade, and orcinol monohydrate, were obtained from Fisher Scientific Company, Fair Lawn, New Jersey.

Cesium chloride, optical grade, was obtained from Harshaw Chemical Company, Hastings on Hudson, New York, and Maywood Chemical Company, Maywood, New Jersey.

DEAE cellulose, reagent grade, was obtained from Brown Company,

North Stratford, New Hampshire. The DEAE was washed with 1 N NaOH and 1 N HCl successively three times, and then washed with deionized water until a constant pH was reached, and then stored in 7 parts deionized water and 1 part 70% ethanol. Before use, the DEAE, was equilibrated with the starting buffer to be used.

Carrier-free P^{32}-orthophosphate was obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee. It was neutralized with NaOH before use. P^{32} was counted in a Tracerlab end-window Geiger counter.

Cell cultures. L cells, strain 929, were grown in suspension (Baltimore and Franklin, 1962a) for the preparation of large quantities of virus for physical-chemical examination. The cells in suspension culture were collected and inoculated with virus when the cell density reached 4-5 x 10^5 cells per ml or 6-7.5 x 10^7 cells per bottle.

Virus. The Dearing strain of reovirus 3 was cloned five times in succession and a stock prepared. The latter was stored at -55° C. Batches of virus were prepared as follows: L cells from suspension cultures were collected by centrifugation, and resuspended in 3.5 ml of reovirus stock containing 2-3 x 10^8 PFU/ml. The ratio of virus to cell was approximately 10:1. After an adsorption period of 2 hours at room temperature, during which the cells were frequently resuspended, growth medium, supplemented with 2% fetal bovine serum, was added to restore the original cell density of 4-5 x 10^5 per ml. The infected cells were then incubated on a roller machine for 36 to 48 hours. The growth of virus in cells in suspension is similar to that in monolayer cultures. After maximal viral growth and release had occurred, the cells were sedimented at 10,000 g for 15 minutes, and the supernate was used for virus purification. Approximately 7 liters of cell culture supernate were prepared per week. This contained virus released from 5 x 10^9 cells and yielded 1-5 mgm of purified virus.

Purification of reovirus. The cell culture supernate was concentrated by ultrafiltration under reduced pressure (Berggård, 1962) to a volume of 250 ml. The concentrated virus suspension was then centrifuged at 78,000 g for 3 hours. To the pellet was added a solution containing 15-30 µg each of deoxyribonuclease, ribonuclease, and chymotrypsin in phosphate buffered saline, pH 7.2, containing .005 M Mg^{++}. The virus is resistant to these enzymes. The pellet was held at 4° C for 4-6 hours,

the virus was resuspended, and then incubated at 37° for 1 hour. The enzyme-treated suspension was then homogenized with half its volume of the fluorocarbon Genetron 113, and the aqueous phase was collected. The final step in the purification, which consisted of equilibrium density centrifugation in CsCl (Meselson et al., 1957), is described in Figure 12.

Analytical methods. DNA was estimated by the diphenylamine reaction as modified by Burton, 1956. A preparation of highly polymerized calf thymus DNA, kindly made available by Dr. Muriel Roger, was used as a standard. RNA was estimated by the orcinol reaction (Mejbaum, 1939). Yeast ribonucleic acid, obtained from Worthington Biochemical Company, and E. coli ribosomal RNA prepared by Mr. George Spyrides of The Rockefeller Institute were used as standards. Protein determinations were performed by the method of Lowry et al., 1951, using Fraction V bovine albumin as a standard. The base ratios were determined by the method of Smith and Markham (1950). Ascending chromatography in tertiary butanol and aqueous hydrochloric acid was carried out on acid-washed Whatman 40 paper for two days. The paper was then dried, the ultraviolet absorbing areas were located, and then eluted with 0.1 N hydrochloric acid. UV absorbance was measured in a Beckman Model DU or in a Carey recording spectrophotometer.

Equilibrium density centrifugation of reovirus

In order to achieve further purification, partially purified reovirus was subjected to CsCl density gradient centrifugation. After a density gradient had been established, a solid main band, about 2.5 mm in width, was seen in the center of the tube. As shown in Figure 12, the bulk of the hemagglutinating activity - a measure of the virus - together with most of the ultraviolet absorbing material, were located in the main band which had an average density of 1.380. In the experiment shown, there was also a small upper band which contained only a very small percentage of the total material and viral activity. In some experiments two small upper bands were seen, in others, none. Another inconstant feature was the presence of a small pellet.

The average density of 14 purified preparations of reovirus in cesium chloride was 1.3827 gm/ml. The average density determined by the equilibrated density gradient columns (Hvidt et al., 1954) was 1.3803. The ratio of viral infectivity to hemagglutinating activity was unchanged

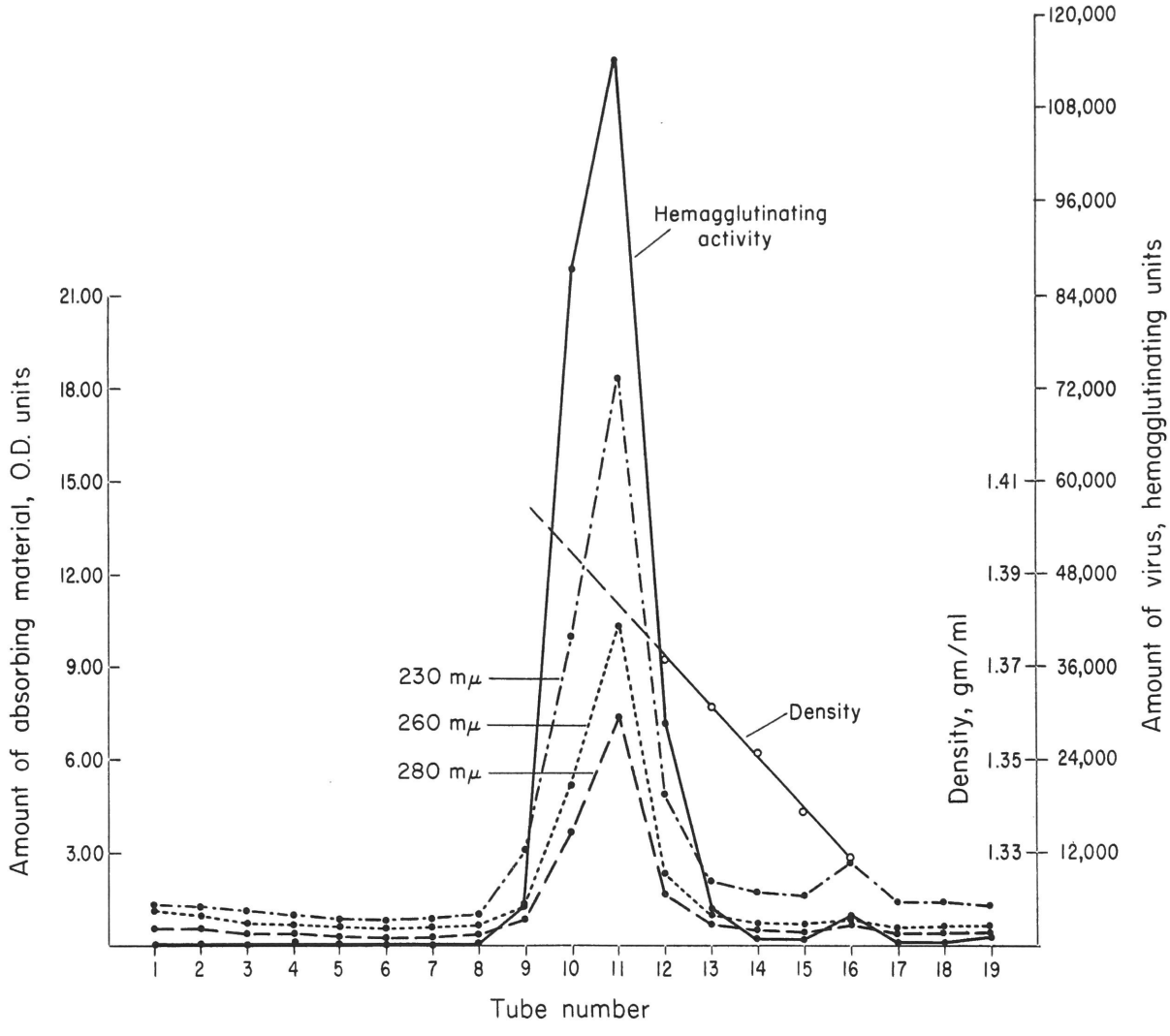


Figure 12. Equilibrium density gradient centrifugation of partially purified reovirus 3. To the enzyme- and fluorocarbon-treated virus suspension was added cesium chloride to an average density of 1.37. The suspension was centrifuged in a swinging bucket rotor of the Spinco Model L centrifuge at 110,000 g for 18 hours at 6° C. Five drops were collected from below into each of the receiving tubes. The material contained in the main virus band was dialyzed extensively and the purified suspension stored at 4° C.

by the purification procedure.

Virus purity

Material from the main band was examined in the electron microscope. The only element seen was virus; no cellular contaminating material was found. The upper bands, which are of less density than the main band, contained cellular material and some virus.

The constancy and reproducibility of the UV absorbancy ratios also indicated a high degree of purity of the virus preparations. As can be seen in Table XV, the mean of the 230/260 absorbancy ratios of various preparations was 1.81 with a range from 1.76 to 1.91; the mean of the 260/280 absorbancy ratios was 1.39 with a range of 1.37 to 1.41. Figure 13 shows the UV absorption spectrum of reovirus. The absorption maximum is at 261 and the minimum at 244 mμ.

The purified virus was further examined in the analytical ultracentrifuge by Dr. David Yphantis. The bulk of the material in the virus suspension, amounting to 60% of the total, traveled as one band. There was one other major component (30% of the total) which preceded the main band in the centrifugal field. This heavier band was heterogeneous and may be attributed to the presence of aggregated virus, since we have observed that the virus has a marked tendency to aggregate. A minor component which followed the main band, probably contained virus particles without cores. Because the only component visible in the electron microscope is virus, it would appear that the explanations which we have offered for the heavier and lighter components are reasonable and in all probability correct.

Chemical composition of reovirus 3

Since our evidence indicated that the virus preparations were essentially pure, chemical analysis was carried out. Table XVI shows the results of determinations of protein and RNA. The mean percent of chemically measured ribonucleic acid in the various preparations was 14.6%, with a range from 13.4 to 16.7%. The diphenylamine reaction was negative. Thus, within the limits of sensitivity of the procedure, there appears to be no deoxyribonucleic acid present. The infectivity of the virus is unaffected by treatment with ether, a fact which suggests that there is no peripheral structural lipid present in the virus particle. No tests

Table XV

Absorbancy Ratios of Reovirus

Preparation	230/260	260/280
A	1.76	1.39
B	1.78	1.40
C	1.91	1.41
D	1.76	1.38
E	1.78	1.38
F	1.78	1.37
G	1.85	1.41
H	1.87	1.41
Mean	1.81	1.39
Range	1.76-1.91	1.37-1.41

The samples were dialyzed extensively against 0.3 M glycine, 0.05 M $MgSO_4$, pH 6.8, and the absorbancies determined in a Beckman DU spectrophotometer.

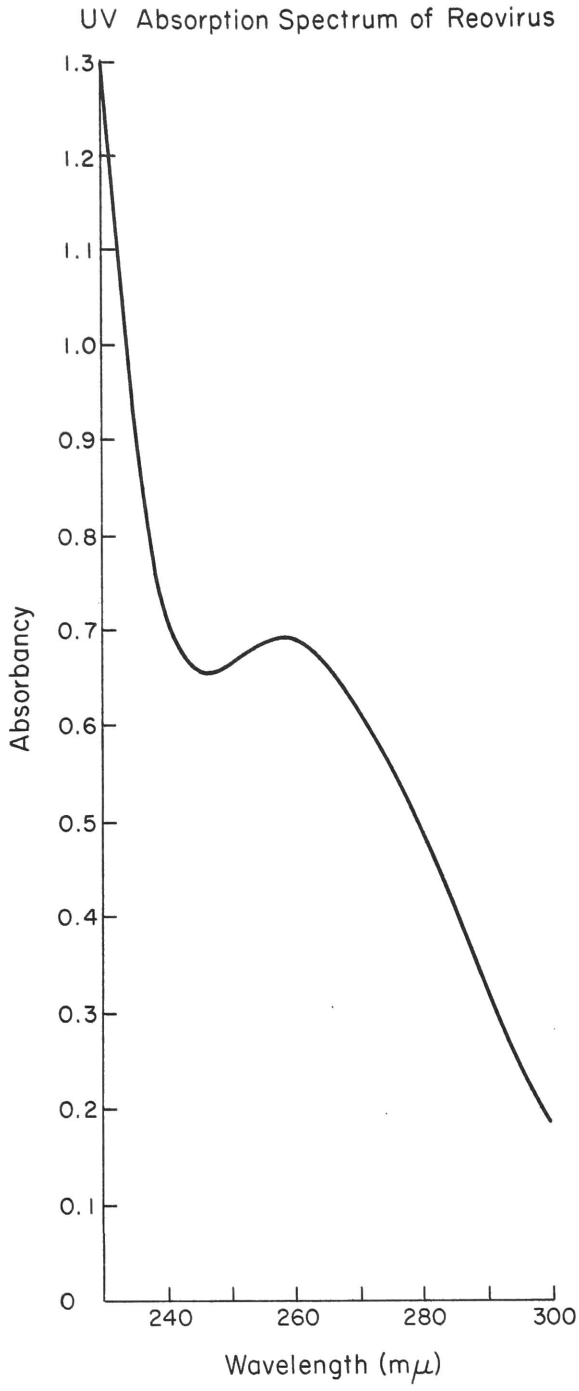


Figure 13. UV absorption spectrum of reovirus in 0.85% NaCl, 0.01 M PO_4, pH 7.2.

Table XVI

Chemical Composition of Reovirus

Preparation	RNA	Protein	RNA + Protein	% RNA
	μg/ml of purified virus suspension			
A[a]	68.4	341	409	16.7
B[a]	38.3	248	286	13.4
C[a]	73.0	400	473	15.4
D[b]	30.0	193	223	13.5
E[b]	39.6	242	282	14.0
F[b]	48.5	280	329	14.7
Mean				14.6

[a] Samples dissolved in deionized water.
[b] Samples dissolved in 0.3 M glycine, 0.05 M $MgSO_4$, pH 6.8.

were done to determine whether carbohydrates are present in the virus other than the ribose of the RNA. All orcinol reactive material is assumed to be contained in the viral RNA.

To arrive at an estimate of the amount of RNA in the virus particle, the mass of virus was determined by sedimentation and diffusion analysis. These experiments were also performed in collaboration with Dr. David Yphantis. The sedimentation coefficient of reovirus was found to be 630 S. An accurate derivation of the diffusion coefficient is difficult because of the number of components which were necessarily present in the purified virus. The diffusion coefficient was found to be 8.3×10^{-8} cm^2 per sec. A minimum estimate of the mass of the virus particle is 70×10^6 molecular weight units. Since the virus contains 14.6% of nucleic acid, a minimum estimate of the mass of RNA in a virus particle is 10.2×10^6 molecular weight units. Examination of reovirus RNA in the analytical ultracentrifuge has shown that it comprises a single molecular species.

Base composition of reovirus RNA

The nucleic acid was extracted from the virus particle with phenol (Gierer and Schramm, 1956) at room temperature. The ultraviolet spectrum is shown in Figure 14. It will be observed that the maximum absorbancy is at 261 and the minimum at 233 mμ. Reovirus RNA was analyzed by the procedure of Smith and Markham (1950) to determine its base composition. After acid hydrolysis and paper chromatography, four spots were found which had the R_F values and absorption spectra of guanine, adenine, cytidylic acid, and uridylic acid. As shown in Table XVII, the mole percent of guanine closely approximates that of cytosine, and the mole percent of adenine approximates that of uracil. The mole percent of G + C is 39.8 of the total. The ratio of purines to pyrimidines is 0.96, and the ratio of A + U to G + C is 1.51. As will be subsequently described, the base composition of a variant strain of reovirus type 3 (Dearing) was also determined and was found to be similar to that of the prototype strain.

Thermal denaturation of reovirus RNA

In order to characterize reovirus RNA further, the heat stability of its secondary structure was determined. Samples of reovirus RNA, dissolved in a medium containing 5×10^{-3} M NaCl, 10^{-3} M PO_4, 10^{-4} M EDTA, pH 7.0, were heated in quartz cuvettes in a thermostatically controlled

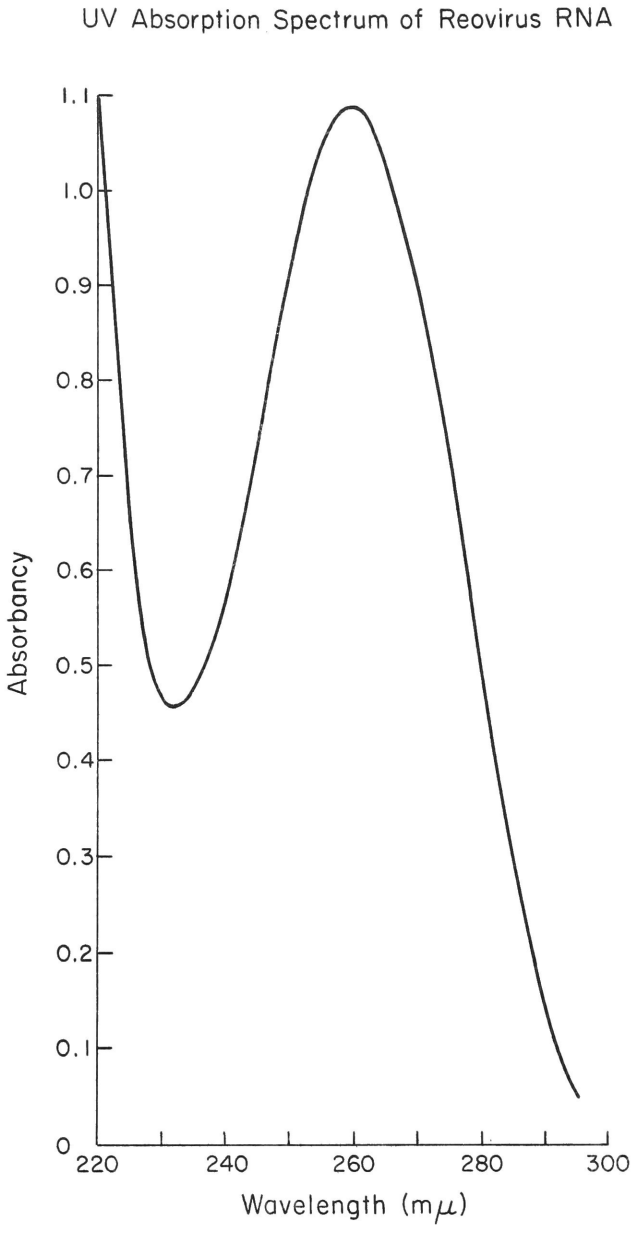


Figure 14. UV absorption spectrum of reovirus RNA in 0.3 M glycine and 0.05 M Mg^{++}, pH 6.8.

Table XVII

Base Composition of Reovirus RNA

Preparation	Bases, mole %			
	G	A	C	U
A	20.7	28.4	22.0	28.8
B	17.9	30.9	18.9	32.2
Mean	19.3	29.7	20.5	30.5

$\frac{A + G}{C + U}$	0.96
$\frac{A + U}{G + C}$	1.51
Mole % G + C	39.8

Beckman spectrophotometer, and the absorbancy at 260 mμ was determined at various temperatures. There was no change until a temperature of 93° was reached when a small increase was observed. To determine the behaviour of reovirus RNA at higher temperatures, samples were heated in stoppered tubes for 10 minutes in a bath containing ethylene glycol and water. They were then cooled rapidly and the absorbancy determined. A maximal hyperchromic effect was obtained at 102° C; heating at 110° C did not result in a further increase in absorbancy.

In Figure 15 is shown the change in absorbancy of reovirus RNA as the temperature was increased from 64 to 102° C. The sample used had already been heated to 83° C in the spectrophotometer and rapidly cooled. As can be seen, the first indication of a steep rise in absorbancy was observed at 94° and the T_m was approximately 99°. Reovirus RNA dissolved in deionized water showed a similar T_m. In three experiments with reovirus RNA dissolved in 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7, the increase in absorbancy began at 86-89° C and the T_m was between 90 and 95°.

Reaction of reovirus RNA with formaldehyde

Formaldehyde is thought to react with the free amino groups of the nucleic acid bases (Fraenkel-Conrat, 1954; Staehelin, 1959; Haselkorn and Doty, 1961; Stollar and Grossman, 1962). When RNA from various sources reacts with formaldehyde, it exhibits both a marked increase in absorbancy at 260 mμ and a shift of the maximum to slightly longer wave lengths. Native DNA, on the other hand, does not show such changes, whereas denatured DNA does.

Figure 16 shows the effect of 1.5% formaldehyde on reovirus RNA dissolved in 0.01 M NaCl, 0.075 M Tris, 0.005 M $MgCl_2$, pH 7.0. The spectra before and after treatment are shown. It may be seen that formaldehyde caused only relatively small changes in the ultraviolet absorption spectrum of reovirus RNA, whereas that of ribosomal RNA was markedly altered. The changes in the spectrum of viral RNA are similar to the minimal changes observed in the spectrum of calf thymus DNA.

It is known that the melting temperature of polynucleotides with intramolecular hydrogen bonds is lowered in the presence of formaldehyde.

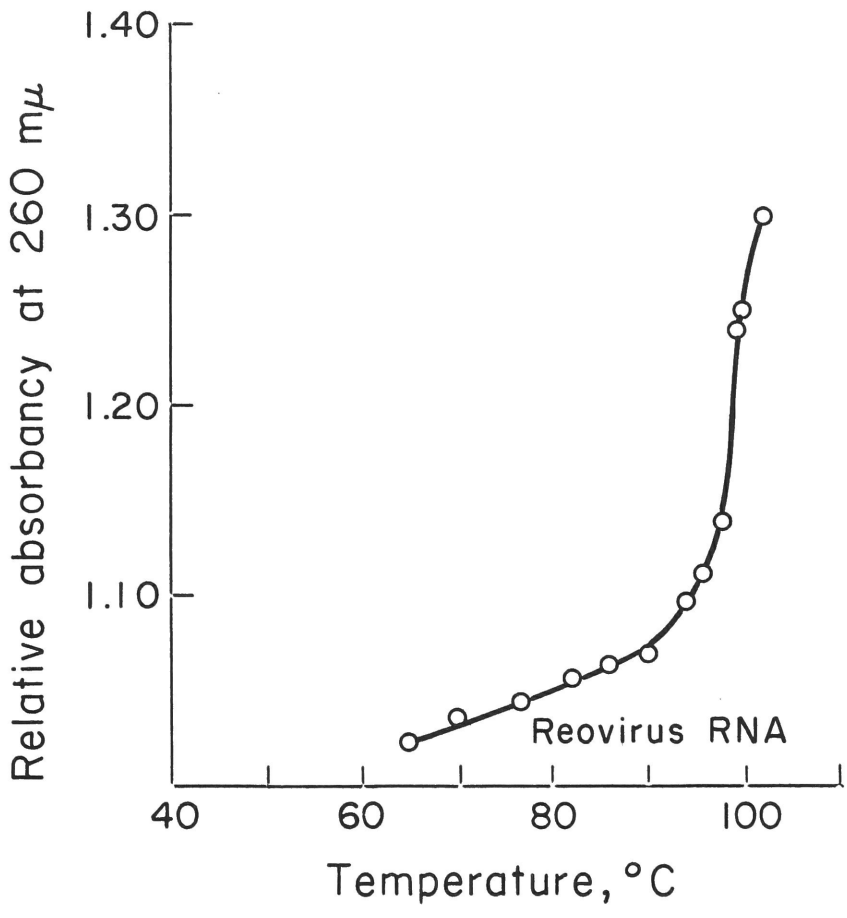


Figure 15. Thermal denaturation of reovirus RNA. Reovirus RNA, 27 μg/ml in 5 x 10^{-3} M NaCl, 1 x 10^{-3} M PO_4, 1 x 10^{-4} M EDTA, pH 7.0, was heated in a stoppered vial at a given temperature for 10 minutes, and then rapidly cooled in ice. The same sample was then heated at the next higher temperature. The small changes in concentration which resulted from evaporation were followed by reweighing sample or measuring its volume and the absorbancies corrected accordingly.

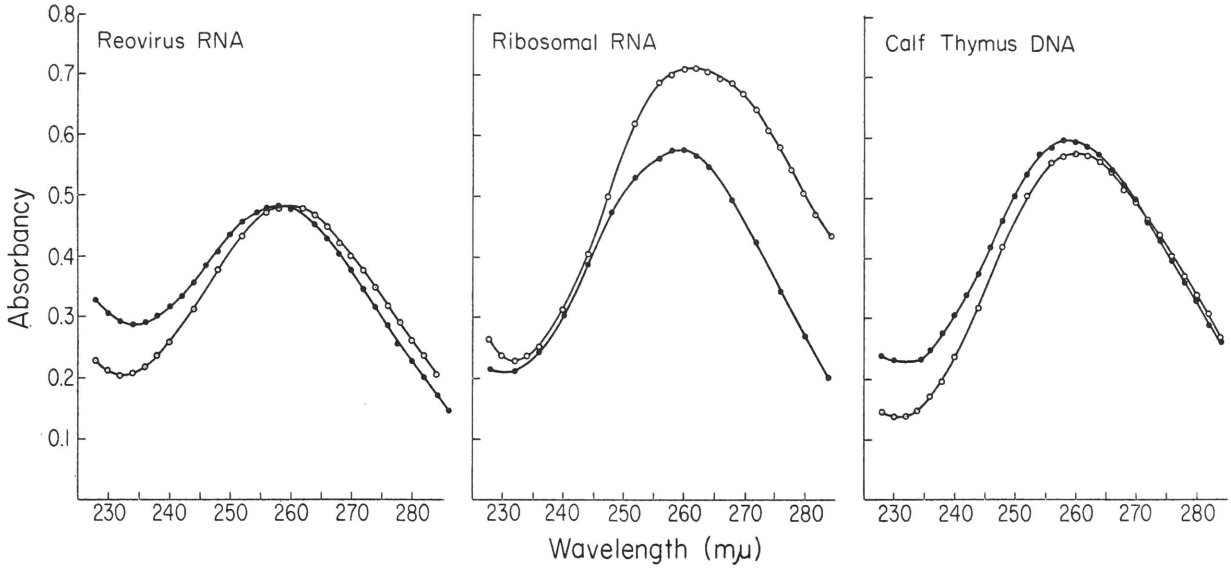


Figure 16. Reaction of reovirus RNA, E. coli ribosomal RNA, and calf thymus DNA with formaldehyde. Samples were dissolved in 1 x 10^{-2} M NaCl, 5 x 10^{-3} M $MgCl_2$, 0.075 M Tris, pH 7.0, and UV absorbancies determined. Formaldehyde was then added to each sample to a final concentration of 1.5%, the pH readjusted to 7.0 and the samples incubated at 32° C. The UV spectrum of the treated samples was determined after 9 hours for reovirus RNA and calf thymus DNA, and after 4 hours for ribosomal RNA. .--. untreated samples; o--o = treated samples.

Figure 17 reveals that when reovirus nucleic acid was heated in the presence of formaldehyde, the hyperchromic effect already became apparent at 65° C.

Effect of ribonuclease on reovirus RNA

Reovirus ribonucleic acid, when exposed for 2 hours to pancreatic ribonuclease (1 μg/ml in 0.01 M NaCl, 0.05 M Tris, 0.005 M $MgCl_2$, pH 7.8) showed no hyperchromic effect either at 260 mμ or at any other wavelength in the ultraviolet region. A control sample of ribosomal RNA showed a marked hyperchromic effect after treatment with RNAase. 85% of the RNAase-treated viral RNA was recovered by alcohol precipitation; 93% of an untreated sample of viral RNA was recovered. Reovirus nucleic acid thus appears to be resistant to the action of pancreatic ribonuclease.

Base composition of Dearing strain variant

As shown previously, the Dearing strain variant is not significantly different from the original Dearing virus in antigenic constitution, size, fine structure, rate of adsorption to L cells, latent period, or cytopathic effects. However, it is less sensitive to specific antibodies and is released to a lesser extent from L cells. The results to be reported below indicate that the base composition of the Dearing strain variant is similar to that of the original prototype strain.

L cells were grown in monolayers in phosphate-free reinforced Eagle's medium containing 5% fetal bovine serum and 30 μc/ml of carrier-free P^{32} orthophosphate for 48 hours. The cultures were then inoculated at a high virus:cell multiplicity with the Dearing strain variant, and incubation at 37° C in the radioactive growth medium was continued. When the cells had degenerated, the fluid and cells were frozen and thawed three times and the suspension centrifuged at 8000 g for 10 minutes. The supernate was then centrifuged at 42000 g at 10° for 2 hours, and the sediment re-suspended in a medium containing DNAase, 16 γ/ml, RNAase, 20 γ/ml, and 0.003 M $MgCl_2$. The suspension was incubated for 12 hours at 4° and for 30 minutes at 37°. To the suspension was then added fetal bovine serum to a final concentration of 5% and it was centrifuged at 80,000 g for 2 hours. The pellet was resuspended in 0.02 M PO_4, pH 7.2, and the sample fractionated on a column of DEAE-cellulose by increasing NaCl concentration. The radioactivity, hemagglutinating activity, and infectivity of the various fractions were determined. Virus first eluted from the column

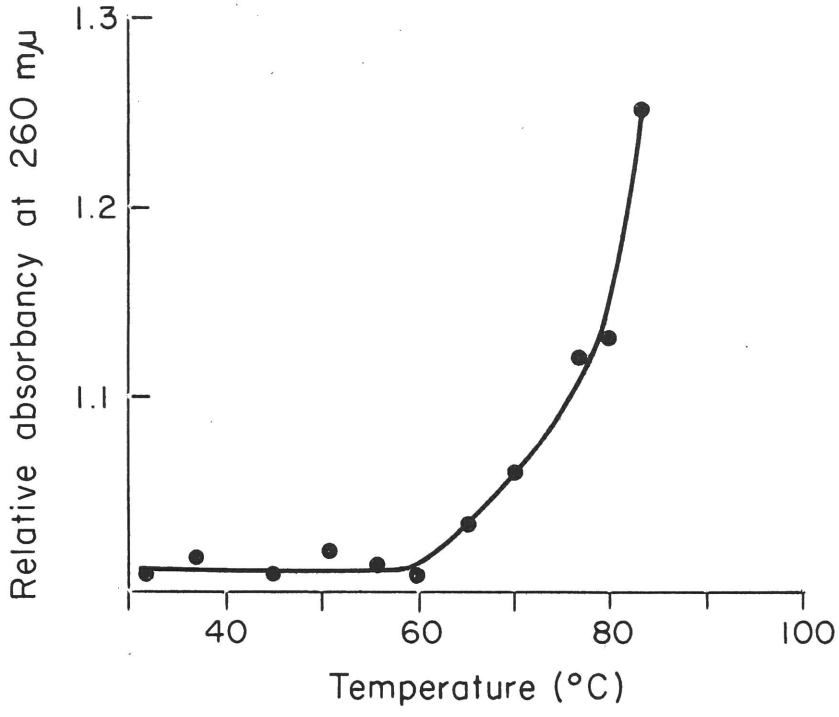


Figure 17. Absorbance of reovirus RNA in 1.5% formaldehyde as a function of temperature. The sample of reovirus RNA which had been treated with 1.5% formaldehyde (cf. Figure 16) was heated in a stoppered vial for 10 minutes at each of the temperatures shown, rapidly cooled in ice and the absorbancy at 260 mμ recorded. Correction was made for any change in concentration resulting from evaporation.

at a salt concentration of 0.3 M. The viral elution pattern showed a sharp simultaneous rise of the three parameters to a maximum, and then a gradual decline. To the eluate showing maximal viral infectivity and radioactivity were added the eluates from neighboring tubes and the suspension was centrifuged at 80,000 g for 2 hours. The pellet was resuspended in a cesium chloride solution of an average density of 1.34 and centrifuged at 100,000 g for 24 hours. No bands were visible because relatively small amounts of virus had been used. Successive drops were collected from below, and assayed for radioactivity and infectivity. The peaks were coincident, and located below the center of the centrifugation tube. The contents of the tubes containing virus were pooled and centrifuged at 80,000 g for 2 hours.

The pellet was resuspended in deionized water. The nucleic acid was extracted from the virus with phenol at 50°. The RNA was hydrolyzed in 1 N KOH at 22° for 22 hours, and then K^+ ions were removed by the procedure of Littlefield and Dunn (1958). Carrier ribonucleotides were spotted with the hydrolysate and the nucleotides were separated by electrophoresis on Whatman no. 3 paper by the procedure of Davidson and Smellie (1952), and Smith (1959). The ultraviolet absorbing areas were located, eluted, and the radioactivity was measured. The base composition of the RNA was estimated from the amount of P^{32} incorporated in the nucleotides.

As can be seen in Table XVIII, the base composition of the Dearing strain variant is similar to that of the original Dearing prototypes strain of reovirus 3. The close similarity in the base compositions is specially noteworthy since the analytical procedures used were different; with the original Dearing virus, the bases were measured by direct chemical determination. In both virus strains, the mole percent of guanine closely approximates that of cytosine, and the mole percent of adenine approximates that of uracil. The mole percent of G + C is 41.2% of the total in the variant, whereas in the original Dearing virus it was found to be 39.8%.

Discussion

From the foregoing it is apparent that reovirus stands apart from most RNA viruses so far examined in that it contains a much larger complement of nucleic acid. The minimum estimate of the molecular weight of reovirus RNA is 10.2×10^6, and it is probable that this value may be considerably larger. In all but three, of the ribonucleic acid-containing viruses, the

Table XVIII

Base Composition of a Variant of Reovirus Type 3 RNA

Virus strain	Bases, mole %			
	G	A	C	U
Dearing strain variant	20.2	29.8	21.0	29.1
Dearing prototype strain	19.3	29.7	20.5	30.5

molecular complement of nucleic acid is of the order of 2×10^6, (Knight, 1954; Frisch-Niggemeyer, 1956; Allison and Burke, 1962b). The exceptions are the RNA-containing phage discovered by Loeb and Zinder, 1961, and two tumor viruses of birds (Allison and Burke, 1962; Bonar and Beard, 1959; Crawford and Crawford, 1961). The molecular complement of the RNA in f_2 phage is 700,000; in avian myeloblastosis virus it has been estimated to be either 4.9×10^6 or 9.8×10^6, and in the Rous sarcoma virus it is approximately 9.5×10^6. The DNA viruses, on the other hand, contain variable amounts of DNA, and in most of these the amount is greater than 10×10^6 molecular weight units (Allison and Burke, 1962).

Reovirus RNA is not only large, but our results indicate that it has a highly ordered secondary structure, namely a double-stranded helix. The complementary values of the base ratios indicate that the nucleic acid does not consist of a single strand folded back upon itself. Positive confirmation of the ordered structure of reovirus RNA will of course have to await X-ray analysis.

The first ribopolynucleotide shown to possess features similar to those of DNA was the T2 complementary (C) RNA (Geiduschek et al., 1962). C-RNA in its biologically active state, however, had none of these features, and its ordered conformation seemed to be an accident of the preparative procedure. Reovirus RNA, on the other hand, probably has an ordered conformation in its native state. Throughout most of the preparative procedure, the nucleic acid was contained within the protein coat of the virus. It is possible that two complementary strands which are not hydrogen-bonded while in the virus particle form a hydrogen-bonded ordered structure during phenol extraction. This appears unlikely, however, since even within the viral inclusion in the cell, the nucleic acid stains orthochromatically green with acridine orange. Furthermore, after pepsin digestion of formalin-fixed thin sections of virus-infected cells, the protein shells of the viral particles slowly disappear and there remains a convoluted, ribonuclease-sensitive, filamentous structure, 25 A in diameter (Bernhard and Tournier, 1962). The diameter is larger than that expected for single-stranded RNA.

Single-stranded ribonucleic acids show a hyperchromic effect over a wide temperature range, consistent with helical regions, short or

defective, or both (Doty, 1962). In contrast, the hyperchromic effect exhibited by reovirus RNA, T2-C-RNA, and the synthetic double-stranded ribonucleotide copolymers occurs only over a narrow temperature range. The melting temperature of 99° of reovirus RNA is surprisingly high. DNA with 40% G + C should have a T_m less than 87° in the same medium (Doty, 1962). The T_m of T2-C-RNA is 63° (Geiduschek et al., 1962). The synthetic double-stranded ribonucleotide copolymers melt at lower temperatures than corresponding DNA's. Double-stranded polyadenylic acid, however, melts at a high temperature (Fresco and Doty, 1957; Rich et al., 1961). The high T_m of reovirus RNA suggests that there may exist special features in its secondary structure which are responsible for the extraordinary thermal stability. There is as yet no evidence for the presence of polyamines, or of metals within the reovirus particle, agents which are known to increase the T_m of polynucleotides (Mahler et al., 1961; Fuwa et al., 1960).

III. RESULTS, cont.

E. Secondary Structure of Wound Tumor Virus RNA

Secondary Structure of Wound Tumor Virus RNA

It is of great interest that a plant virus which multiplies and causes wound tumors in a variety of plants is similar to reovirus in size and structure (Brakke et al., 1954; Bils and Hall, 1962). The wound tumor virus and reovirus both measure about 700 A in diameter and both possess 92 structural subunits (Vasquez and Tournier, 1962; Mayor and Melnick, 1962; Bils and Hall, 1962).

Isolation of wound tumor virus from agallian leafhoppers captured in the neighborhood of Washington, D.C., was first reported by Black in 1944. The virus which could be transmitted only by certain species of agallian leafhoppers, produced pathological changes in more than 43 species of plants distributed in 20 different plant families (Black, 1945). The changes consisted of enlargement of veins, outgrowths on the underside of leaves, some crinkling of leaves, and tumors, usually on the roots; only in sweet clover were tumors also produced on the stem (Black, 1945). The size, shape, and location of the tumors in sweet clover seemed to be host controlled (Black, 1951). The geographical distribution of wound tumor virus is unknown.

The virus is acquired by the leafhopper while feeding on an infected plant. A minimum incubation of 13-15 days at optimum temperatures is required before the insects are able to transmit the virus to plants (Black, 1958). Wound tumor virus multiplies in the insect vector without any overt deleterious effects (Black and Brakke, 1952; Maramorosch, 1955; Black, 1958), and it is transmitted transovarially to about 2% of progeny (Black, 1953). Disease in the plants is visible usually 2 weeks after the feeding by an infected leafhopper (Black, 1958).

Brakke et al. 1954, showed that virus isolated from plants and insects was similar in size and structure when viewed in the electron microscope. Virus from the two sources was also similar in sedimentation behaviour and in antigenic constitution. Virus from plants and insects retained infectivity after 10 minutes at 50°, but was completely inactivated after 10 minutes at 60°; it was stable for 1 hour at 0° at pH 4-9 (Brakke et al., 1953).

Histochemical examination of virus-induced tumors in sweet clover revealed cytoplasmic inclusions, termed spherules (Black, 1946). The chemical constitution of the spherules could not be determined unequivocally, but they contained no Feulgen-positive material. They resisted the action of proteases, and stained red with the methyl green-pyronin stain (Littau and Black, 1952). In general, the staining characteristics of the inclusions were similar to those of the nucleolus. Virus-specific antigenic material, stained with fluorescein-labeled antibody, was seen only in the cytoplasm (Nagaraj and Black, 1961).

According to Bils and Hall (1962), Black has found that wound tumor virus does contain RNA and that it possesses a sedimentation coefficient of about 510 S. This figure is lower than the value of 600 S reported previously by Brakke et al. (1954), and it is also lower than the value of 630 S obtained in the present study for reovirus. Wound tumor virus particles contain a nucleoid whose volume has been estimated to be 20% of the volume of the virus (Bils and Hall, 1962). When wound tumor virus was placed in a medium of low ionic strength, it released long strands about 30 A in diameter. Very few strands remained after heating the preparation at 70° for 15 minutes. After 15 minutes' exposure to trypsin, 0.1 mgm/ml, at 37°, most of the strands broke and some were puddled; after treatment for 30 minutes, few strands remained. Bils and Hall concluded that the material in the strands was a ribonucleoprotein or an unusual RNA.

The structural similarities between wound tumor and reo viruses suggested that the nucleic acid of wound tumor may also be a double-stranded polyribonucleotide and experiments were undertaken to determine the secondary structure of the RNA of this plant virus.

Materials and Methods

Alundum. Crystalline alumina, 60 mesh (blue label), free of surface alkali, was obtained from Norton Company, Worcester, Mass.

Virus. Cuttings from clone C_{10} of infected sweet clover were kindly made available by Dr. L.M. Black. Clover plants were grown in soil in a greenhouse whose temperature varied between 75 and 80°. The light was on for 12-14 hours each day. After 2 months, since only a few small tumors were visible on the plant stems, the stems were injured in numerous places

with a pin. One month later, numerous tumors had formed and were removed from the stem with a knife. Three to four grams of tumor material was removed on each of two occasions and frozen at -28°. The tumors were then ground with alundum in phosphate buffered saline to a thick paste. The homogenate was diluted with PBS to give a 10% suspension, filtered through fine gauze, and then centrifuged at 10,000 g. The supernate which contained the virus was subjected to the same purification procedure as described above for the purification of reovirus, including centrifugation, enzymatic and fluorocarbon treatments, and banding in a cesium chloride gradient. The density of the main band was 1.42. A preparation which had not been treated with enzymes or fluorocarbon, but had been banded in the cesium chloride gradient, was examined by Dr. Samuel Dales and was found to contain wound tumor virus particles and minimal amounts of cellular contaminating material.

UV absorption spectrum of wound tumor virus and its nucleic acid

Figure 18 shows that the absorption maximum of wound tumor virus is at 255 and the minimum at 248 mμ. The UV absorbancy ratios, 230/260 and 260/280, are 1.89 and 1.40, respectively, values closely similar to those obtained for reovirus. The maximum absorbancy of the virus RNA is at 258 and the minimum at 237 mμ.

Thermal denaturation of wound tumor virus nucleic acid

Figure 19 shows that on heating to 93.3° C the nucleic acid extracted from wound tumor virus and dissolved in 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0, exhibited a hyperchromic effect of 36%. Absorbance began to increase sharply at 83° and the T_m was 89° in this medium.

Discussion

Evidence has been obtained that the nucleic acid of wound tumor virus has a highly ordered secondary structure similar to that of reovirus RNA, namely a double-stranded helix.

There is a third virus which shares structural characteristics with reo and wound tumor viruses. The rice dwarf virus produces a dwarfing disease in rice plants and multiplies both in the plant and in its vector (Fukushi, 1962), again the leafhopper. The size of the rice dwarf virus particles is 75 mμ and the number of the structural subunits may be similar

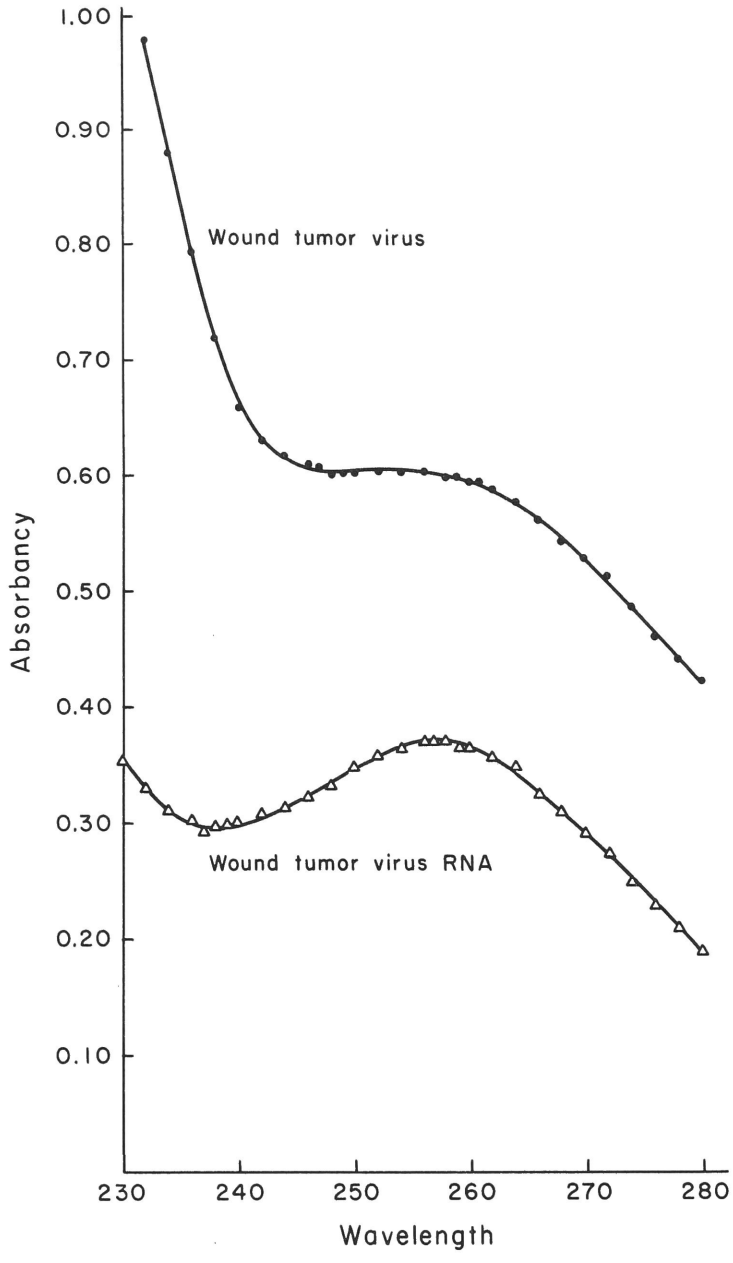


Figure 18. UV absorption spectrum of wound tumor virus and its nucleic acid in 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.

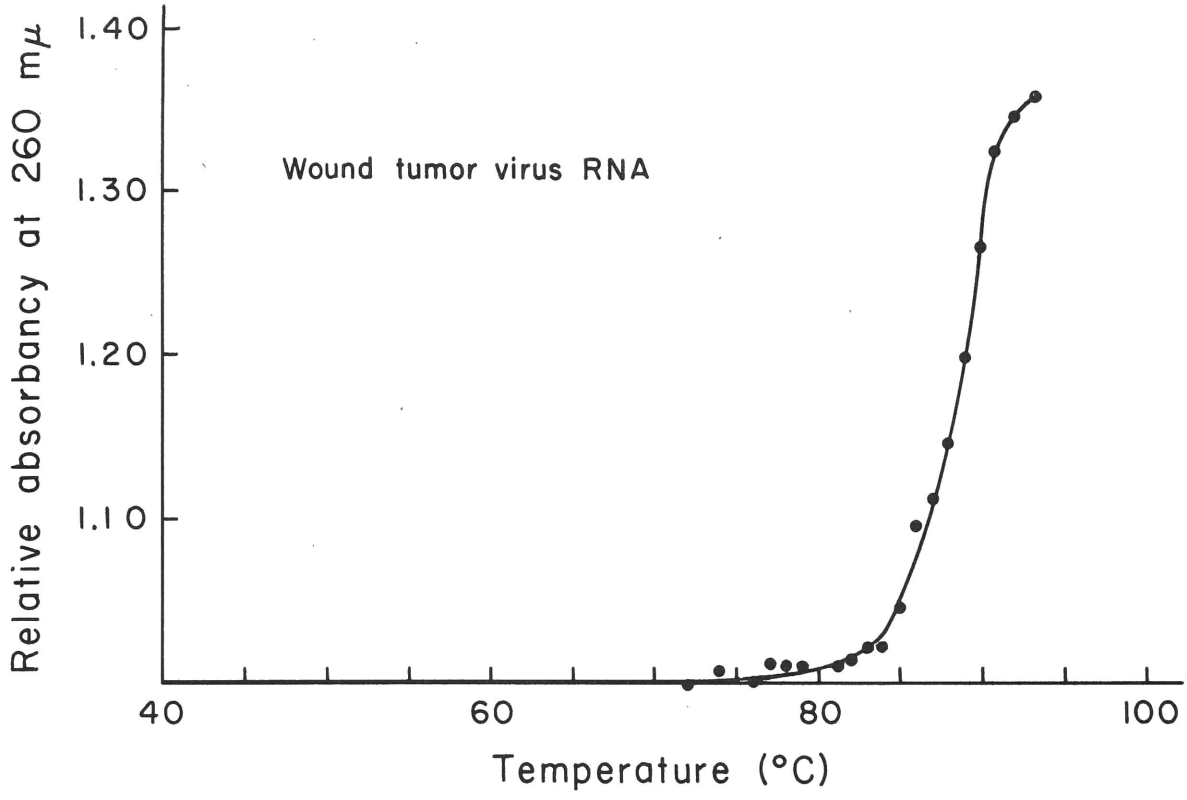


Figure 19. Thermal denaturation of wound tumor virus RNA. Wound tumor virus RNA in 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7 was heated in stoppered quartz cuvettes in a thermostatically controlled Beckman spectrophotometer and absorbancy at 260 mμ was determined at various temperatures. Correction was made for any change in concentration resulting from evaporation.

to that previously described for reo and wound tumor viruses, i.e., 92. The rice dwarf virus multiplies only in the cytoplasm of cells. Virus particles form crystalline arrays and inclusion bodies are seen in infected cells. The virus not only produces disease in the plant, but also causes degenerative changes in the cells of the insect vector.

The wound tumor virus and the rice dwarf virus are two of the very few viruses known to transcend the plant and animal kingdoms (Maramorosch, 1955; Black, 1959). The pathological changes produced by the viruses in their various hosts are different; in fact, the wound tumor virus does not adversely affect its insect vector. If it is shown that the nucleic acid of rice dwarf virus is also a double-stranded helical polyribonucleotide, then it would appear that this relatively large and unusual structure may be endowed with special characteristics which permit replication in widely different hosts.

IV. GENERAL DISCUSSION

GENERAL DISCUSSION

Reo, wound tumor, and rice dwarf viruses are all of similar size and possess a similar surface structure. Reovirus contains a large amount of genetic material, which is RNA. From the volume which is occupied by the nucleoid, it may be estimated that wound tumor and rice dwarf viruses also contain a large amount of nucleic acid. The secondary structure of the nucleic acid of reo and wound tumor viruses appears to be a double-stranded helix; that of rice dwarf virus is unknown. However, if the size and structure of the protein coat is determined by the amount and structure of nucleic acid which it encloses, it follows that the nucleic acid component of rice dwarf virus may also be a double-stranded polyribonucleotide of large size.

Horne and Wildy (1962) considered how many pentagonal and hexagonal surface subunits or capsomeres would be required to give bodies with 5:3:2 symmetry. At least four series of bodies exist satisfying necessary geometric requirements. As had been noted by Buckminster Fuller (1960), the first two follow the general formula of $10x(n-1)+2$, where x could take the value of 1 or 3. When x, an empirical number, is one, a series of icosahedral bodies is formed in which the subunits on the edges are shared between adjacent faces; n is the number of structural subunits along an edge of the body. The solutions when x is 1 and n is varied from 2 to 10 give values of 12, 42, 92, 162, 252, 362, 492, 642, and 812. Viruses with several of the predicted numbers of capsomeres were soon identified, and all were found to contain DNA: phi x 174 with 12 capsomeres; polyoma, Shope papilloma, and SV40 with 42; herpes simplex with 162; adenovirus with 252; and tipula iridescent virus with 812. Viruses with 92 capsomeres were not found until reo and wound tumor viruses were examined. They, however, contain RNA. A common feature of this series of viruses that does remain is that the genetic material of the viruses in the series has a highly ordered secondary structure.

The only apparent exception is the first and smallest member of the series. The small bacteriophage phi x 174 has been thought to contain single-stranded DNA (Sinsheimer, 1959; Sinsheimer et al., 1962). The size of this DNA is 1.7×10^6 in molecular weight units. On examination of

platinum shadowed preparations of the nucleic acid released from the virus particle, Maclean and Hall (1962) observed long rigid strands 10-40 A in diameter. The strands were disrupted by treatment with DNAase and by heating to 80°. Despite the resistance of the secondary structure of this nucleic acid to proteolytic enzymes, the authors considered the strands to be DNA, irregularly coated with protein. The non-complementarity of the base ratios notwithstanding, Cavalieri and Rosenberg (1962) express doubts that the single-stranded nature of this nucleic acid has been as yet unequivocally established. The nucleic acid of phi x 174 behaves as denatured double-stranded DNA, and no conclusive evidence has been presented that on denaturation the nucleic acid does actually separate into two single and separate strands (Cavalieri and Rosenberg, 1962).

Most RNA viruses possess a complement of RNA of about 2×10^6 molecular weight units and are known to be single-stranded (Allison and Burke, 1962; Montagnier and Sanders, 1963). The base ratios of EMC, entero, and influenza viruses are not complementary (Schaffer, 1959; Holland, 1962; Kerr et al., 1962). Viruses belonging to these groups and to the arbor-virus group have not been shown to cause other than degenerative changes in infected cells. These viruses in general have a short latent period and multiply rapidly. In cells infected with Mengo- or poliovirus, the DNA-dependent RNA polymerase becomes inhibited and the synthesis of cellular RNA and proteins is curtailed (Franklin and Baltimore, 1962; Holland, 1962, 1963; Darnell, 1962). Furthermore, a virus-induced RNA polymerase appears which may be the enzyme system responsible for virus RNA synthesis (Baltimore and Franklin, 1962b).

It has been postulated that single-stranded RNA cannot be the repository of stored information in warm blooded animals, since it does not have the necessary stability at 37° (Eigner et al., 1961). The molecular half life, that is the time for the molecular weight of a polynucleotide of 10,000 units to be halved, was found to be 15 hours for single-stranded RNA, and 10 days for single-stranded DNA at 37°. No values were given for double-stranded DNA, but presumably this configuration is much more stable. The mutability of small RNA viruses and the relative stability of DNA viruses are well known.

On the basis of what is known about the biology of virus-induced tumors, it may be postulated that tumor viruses have at least two fundamental

characteristics. Tumor viruses must be able to allow host-controlled syntheses to continue, and their nucleic acid must have the required stability to heat, nucleases, and other denaturating agents to assure the presence of the viral genetic material for a considerable period of time. Continued presence of virus-specific elements has been demonstrated in all the virus-induced tumors save for polyoma in which case all attempts to prove their continued presence have failed (Temin and Rubin, 1959; Ito, 1962; Baluda, 1962; Sabin and Koch, 1963; Vogt and Dulbecco, 1960).

Viruses known to produce tumors have been shown to contain either DNA or RNA. The DNA of tumor-producing viruses has been shown to be double-stranded in all cases (Watson and Littlefield, 1960; Weil, 1962; Green, 1962; Joklik, 1962). To the list of viruses with double-stranded DNA commonly considered as tumor viruses can be added adenovirus, types 12 and 18 (Trentin et al., 1962; Huebner et al., 1962) and under special circumstances vaccinia (Duran-Reynals, 1958). The secondary structure of the RNA of two tumor viruses of birds has not as yet been determined, but the amount of genetic material has been shown to be considerably larger than the usual 2×10^6 molecular weight units. It may be that the double-stranded helical configuration not only may offer stability to denaturating agents, but allow the intercellular transfer in one molecule of a relatively large amount of genetic information which may be a necessary requirement for tumor induction.

Reovirus RNA has a very high T_m, higher than that reported for any other polyribonucleotide, natural or synthetic. There is no evidence as yet in reovirus RNA of agents known to stabilize polynucleotides and to increase thereby their denaturation temperature. X-ray diffraction studies have shown that synthetic double-stranded polyribonucleotides have a secondary structure similar to that of double-stranded DNA, and their X-ray diffraction pattern can be best explained by hydrogen bonding between the bases arranged on the inside of the polyribose phosphate chains with slight tilting from the perpendicular of the planes of the bases (Wilkins, 1961). The presence of the hydroxyl group on the carbon 2 of ribose apparently allows for hydrogen bonding between this group and the neighboring phosphate group. Reovirus appears to have the necessary stability to be a tumor virus, and a closely similar virus, the wound tumor virus, is in fact

tumorigenic in a susceptible host, the sweet clover plant.

Reovirus does not grossly affect the synthesis of RNA and proteins in infected cells, but does inhibit DNA metabolism, suggesting a specific effect on the genetic apparatus of the cell. Spendlove (personal communication) has found that in reovirus-infected cells undergoing mitosis, the viral antigen was associated with the mitotic apparatus of the host cells. It is possible that virus-specific material may physically interfere with the equal distribution of cellular genetic material during division.

Native DNA is a relatively poor primer for the in vitro DNA-dependent DNA polymerase reaction in which deoxyribonucleotide triphosphates are incorporated into a new DNA of high molecular weight and with base ratios similar to that of the primer DNA used (Kornberg, 1961). Denaturation of the primer DNA by heat or DNAase appears to increase its activity in this reaction (Hurwitz et al., 1962). Denaturation of DNA does not necessarily mean separation into single strands; the primer DNA may serve in a partially separated state (Cavalieri and Rosenberg, 1962). On the other hand, heated DNA is only 50% as effective as native DNA in priming the DNA-dependent RNA polymerase reaction in which ribonucleotide triphosphates are incorporated into a messenger, or complementary RNA (C-RNA), with base ratios complementary to that of the primer DNA. Also, Wood and Berg (1962) and Doerfler et al. (1962) have shown that the RNA formed in the presence of denatured DNA is completely inactive in stimulating amino acid incorporation into proteins. It is of interest to note that "single-stranded" phi x 174 DNA passes through a "double-stranded" stage in its replicative cycle (Sinsheimer et al., 1962).

It cannot be predicted whether reovirus RNA uses the normal cellular DNA polymerase to replicate itself, and the cellular RNA polymerase to make an hypothetical C-RNA, or whether new virus-induced enzymes serve in reovirus biosynthesis. It is also possible that some cellular enzymes, and some new virus-induced enzymes are utilized. Lee-Huang and Cavalieri (1963) have shown that double-stranded polyribonucleotides can serve as a "template" for incorporation of deoxyribonucleotides triphosphates into an acid insoluble, double-stranded product using the enzyme DNA polymerase. They did not however explore the suitability of ribonucleotide triphosphates as precursors.

It would not be expected that reovirus RNA in the form of a double-stranded helix would be an efficient messenger in a protein-synthesizing system. In fact, order in the secondary structure of synthetic polyribonucleotides is inhibitory to their capacity to stimulate the incorporation of amino acids into polypeptides (Nirenberg and Matthaei, 1961; Singer et al., 1963). It may be predicted that either a new single-stranded C-RNA is made by reovirus which then can act as a template in the synthesis of protein components of the virus, or that reovirus RNA itself must unwind during the latent period in order to act as an efficient template for the synthesis of virus-specific proteins.

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