*ANIMAL AND PLANT VIRUSES WITH DOUBLE-HELICAL RNA**

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Reoviruses[1, 2] possess outstanding physical and biological properties. The viral genetic material is an extraordinarily stable double-helical RNA[3, 4] of an estimated molecular weight in excess of 10×10^6. The viruses are more widely distributed among animal species, including man, than any other virus known.[5–7] Antibodies to reoviruses have been found in all vertebrates, except whales.[6] Reovirus is a virus in search of disease; no definite association between the virus and disease has been found in man, monkeys, cattle, or dogs.[5–7]

That a plant virus, the wound tumor virus, shares a number of properties with reovirus has aroused speculations concerning the relationship between the two.[3, 8] The plant virus, which multiplies and causes disease in a great variety of plants and tumors in some,[9] is similar to reovirus in size and surface structure. The wound tumor virus and reovirus both measure approximately 700 A in diameter and the capsid of each possesses 92 morphological units.[5, 10–13] In their interaction with cells, both wound tumor virus and reovirus produce characteristic cytoplasmic inclusion bodies which contain virus particles.[5, 14–16] There is no structural evidence of nuclear involvement in the growth of either virus.

Wound tumor virus was first isolated from leafhoppers in which host it also multiplies.[10] The leafhoppers can transmit the virus to more than 43 species of plants distributed in 20 different plant families, including sweet clover and crimson clover, chrysanthemums, and sorrel.[9] Virus isolated from plants and insects is similar in size, structure, sedimentation behavior, and antigenic constitution.[10]

The structural similarities between wound tumor virus and reoviruses suggested that the nucleic acid of wound tumor virus might also be a double-stranded polyribonucleotide, and our preliminary results indicated that this is indeed the case.[3] We have now obtained additional evidence which supports this conclusion. Furthermore, we have found that wound tumor virus RNA contains less G + C and has a lower melting temperature than reovirus RNA.

One of the intriguing biological questions is whether reoviruses—alone or in conjunction with physical or chemical agents—cause tumors in man and animals. There is no answer to this question at present; however, we have been stimulated to consider what the fundamental and necessary properties of the genetic material of tumor viruses might be. We propose that double-strandedness of the nucleic acid, assuring stability of the genetic information, may be one such property.

Materials and Methods.—Cuttings from clone C10 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 hr 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 4 gm of tumor material were removed on each of two occasions from the stems, and 500 gm from the roots on one occasion; all the material was

frozen at −28°. The tumors were washed with running water and then ground with alundum in phosphate-buffered saline (PBS) 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 described previously for reovirus,[3] including centrifugation, enzymatic and fluorocarbon treatments, and banding in a cesium chloride density gradient. The main band had an average density of 1.411. 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 in the electron microscope and was found to contain characteristic virus particles and minimal amounts of cellular contaminating material.

Results.—Base composition of wound tumor virus RNA: The nucleic acid was extracted from the virus particles with phenol at room temperature.[17] The maximum absorbancy of the virus RNA was at 261 and the minimum at 235 mμ. The RNA was analyzed by the procedure of Smith and Markham[18] to determine its base composition. As shown in Table 1, the mole per cent of guanine closely

TABLE 1

BASE COMPOSITION OF WOUND TUMOR VIRUS AND REOVIRUS RNA

Sample	Wound Tumor Virus RNA Bases (mole %) G	A	C	U	G + C
A	18.4	30.2	19.3	32.2	37.7
B	18.8	31.9	18.9	30.4	37.7
Mean	18.6	31.1	19.1	31.3	37.7
		Reovirus type 3 RNA			
Mean value*	22.3	28.0	22.0	27.9	44.3

* Based on 5 analyses on 4 preparations.

approximates that of cytosine, and the mole per cent of adenine approximates that of uracil. The mole per cent of G + C is 38 per cent of total. Thus far, we have had available enough wound tumor virus RNA for only two analyses on samples obtained from the same starting material but purified separately. The results on the two samples agreed closely, as shown in Table 1.

Further results on the base composition of five samples of reovirus type 3 have resulted in a refinement of the G + C value. The new mean value of 44 per cent is similar to the value of 42.7 per cent reported previously on the first preparation examined. For reasons unknown, the second of the two preparations examined previously gave a much lower G + C content (36.8%) than all other preparations of reovirus which have been examined. Thus, the G + C content of wound tumor virus RNA appears to be significantly lower than that of reovirus RNA.

Thermal denaturation of wound tumor virus RNA: Figure 1 shows that, on heating to 95° in a medium of 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7.0, wound tumor virus RNA exhibited a hyperchromic effect of 27 per cent. Absorbance began to increase at 87°, and the T_m of 90° was similar to that reported previously.[3] A third preparation of wound tumor virus RNA melted at a slightly higher temperature, namely 92°. The total increase in absorbancy observed in different experiments has varied between 27 and 36 per cent.

In two experiments with wound tumor virus RNA, parallel observations were made on reovirus RNA in the same sodium chloride-citrate medium. Reovirus

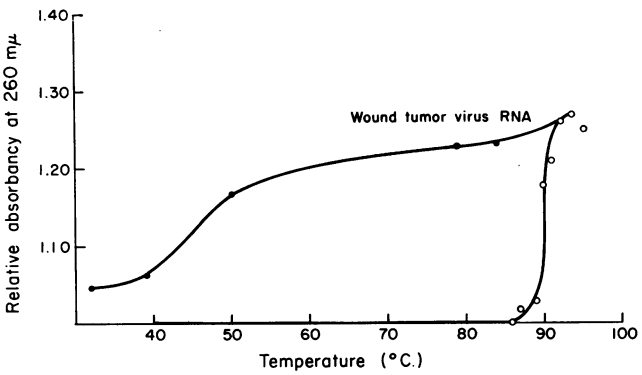

FIG. 1.—Thermal denaturation of wound tumor virus RNA. Wound tumor virus RNA in 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7.0 was heated in a quartz cuvette in a thermostatically controlled Beckman spectrophotometer, and absorbancy at 260 mμ was determined at various temperatures (O—O). After denaturation was complete, the sample was allowed to cool slowly to 31° over a 2-hr period (•—•).

RNA began to melt at a temperature about 3° higher than wound tumor virus RNA. On slow cooling, both the wound tumor virus RNA and the partially melted reovirus RNA regained almost completely their original hypochromicity. In the sodium chloride-citrate medium the T_m of reovirus RNA[3] is approximately 3° higher than that of wound tumor virus RNA.

Lack of immunological relationship between wound tumor virus and reoviruses: With the obvious similarity in the secondary structures of the genetic material of these two viruses, experiments were performed to determine if there were also immunological similarities in their protein coat in addition to the reported similarity in capsomeric number. Immune sera were prepared by intravenous injection of the viruses two times into rabbits at two-week intervals; the sera were collected two weeks after the second injection. Purified wound tumor virus and a lysate of reovirus-infected L cells were used as antigens. The sera were inactivated by heating at 56° for 30 min. Complement fixation was performed by the method recommended by the Viral and Rickettsial Disease Laboratory of the California State Department of Public Health. The sera were further treated with *V. cholerae* filtrate overnight at 37° prior to testing their inhibitory activity on viral hemagglutination and plaque formation.

As can be seen in Table 2, despite the strong fixation of complement by all three prototype strains of reovirus in the presence of immune reovirus 3 serum, there was no cross reaction when wound tumor virus was used as the antigen. Immune serum

TABLE 2

LACK OF IMMUNOLOGICAL RELATIONSHIP BETWEEN WOUND TUMOR VIRUS AND REOVIRUSES

	Complement Fixation Titer			
Antigen	Preimmune serum	Wound tumor virus immune serum	Preimmune serum	Reovirus 3 immune serum
Wound tumor virus	16	64	16	8
Reovirus 1 (Lang)	8	8	<8	>256
Reovirus 2 (Amy)	8	8	<8	128
Reovirus 3 (Dearing)	8	<8	<8	>256

against wound tumor virus had a complement fixation titer of 64 with tumor virus as the antigen; the titer of the preimmune serum was 16. There was also no evidence of immunological cross relationship when the three prototype strains of reovirus and wound tumor virus immune serum were reacted. Furthermore, hemagglutination inhibition and neutralization tests gave no evidence of antibodies against any of the three prototype strains of reovirus in wound tumor virus immune serum.

Thus, we have found no evidence of an immunologic relationship between any of the three types of reovirus and wound tumor virus. While this manuscript was in preparation, a report appeared by Streissle and Maramorosch[8] that there did exist a common complement-fixing antigen in wound tumor and reoviruses. The results which were presented are, however, not convincing. The titers of reovirus preparations employed as antigen in complement fixation tests were unusually low. In addition, to evaluate the significance of complement fixation titers obtained with reoviruses and wound tumor virus hyperimmune serum, the titers were compared with those obtained with pooled normal rabbit serum, rather than preimmune serum from the rabbits from which immune serum was obtained. Because of the ubiquity of reoviruses and their ability to infect rabbits, results of complement fixation lose some of their significance when the preimmune and immune sera are not from the same animal.

Experiments on interaction of wound tumor virus with mammalian cells: Wound tumor virus, purified as described above and dialyzed against phosphate-buffered saline, did not agglutinate human, bovine, guinea pig, or rat erythrocytes either at room temperature or at 4°, nor did it agglutinate chick erythrocytes. Inoculation of the virus suspension onto monolayers of monkey kidney cells or mouse fibroblasts (L929) and passage serially three times over a 41-day period produced no evident cytopathic effects on the monolayers. Subcutaneous and intranasal inoculation of 24-hour-old mice and hamsters with wound tumor virus produced no overt illness during $5^1/_2$ months of observation.

Discussion.—We have obtained evidence that the ribonucleic acids of wound tumor virus and of reovirus have a highly ordered secondary structure, namely, a double-stranded helix. The amount of nucleic acid per reovirus particle is greater than 10×10^6. It is probable, from the volume available to the nucleic acid in the virus particle,[11] that the amount of RNA in wound tumor virus is similarly large. Reo- and wound tumor viruses thus differ from many RNA viruses, all of which possess a complement of about 2×10^6 molecular weight units of single-stranded RNA.[19, 20] Polio- and influenza viruses represent well-studied examples of such cytocidal RNA viruses, whose growth is characterized by a short latent period, followed by a rapid increase in virus. It is of great interest that in contrast to these agents, two tumor viruses of birds contain RNA in an amount considerably larger than the usual 2×10^6 molecular weight units.[19, 21, 22] Whether the RNA of these avian tumor viruses, Rous sarcoma and avian myeloblastosis, is double- or single-stranded, remains to be determined.

It has been suggested that viruses which possess a number of common properties may be considered as related, and that such a conclusion is valid apart from considerations of evolutionary relatedness.[23] On this basis reo- and wound tumor viruses, although they affect widely different hosts, are related agents. It has been

reported that these two viruses have a common complement-fixing antigen.[8] If this were so, evolutionary relatedness would appear likely. However, in the present study we found no evidence of any serological relationship between wound tumor virus and any of the three immunological types of reovirus. Moreover, wound tumor virus failed to agglutinate a variety of erythrocytes and to cause cytopathic effects in mammalian cells. It is not surprising that the base compositions of these two viruses are different. The mole per cent of G + C in wound tumor virus RNA is 38, whereas in reovirus it is 44. Furthermore, the melting temperature of wound tumor virus RNA is lower than that of reovirus RNA, probably reflecting the lower G + C content of the former.

There is a third virus which shares structural and biological 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 insect vector,[24] again the leafhopper. The size of the rice dwarf virus particles is 750 A and the number of structural subunits may be similar to that previously described for reo- and wound tumor viruses, i.e., 92. Like reo- and wound tumor viruses, the rice dwarf virus appears to multiply only in the cytoplasm of the infected cell. Virus particles form crystalline arrays and inclusion bodies are seen. The wound tumor virus and the rice dwarf virus are two of the few viruses known to bridge the plant and animal kingdoms.[25] The pathological changes produced by the viruses in their various hosts are different; indeed, the wound tumor virus does not adversely affect its insect vector,[26] whereas the rice dwarf virus does.[24] If it is shown that the nucleic acid of rice dwarf virus is also a double-helical polyribonucleotide, this would provide additional evidence that this relatively large and unusual structure may be endowed with special characteristics which permit replication in widely different hosts.

The fact that wound tumor virus is capable of causing tumors raises the question whether reovirus may also be oncogenic. The answer is not yet available, and the problem may be complex. Although the wound tumor disease in plants is induced by a virus, the development of tumors in a host is largely limited to areas of irritation.[26, 27] As emphasized by Braun,[27] a host may be systemically infected with the virus and yet no tumor is produced unless cells are triggered to divide in response to injury. Furthermore, the genetic constitution of the host plays an important role in the expression of this disease.[28] These observations quite obviously provide guidelines for investigation of the possible tumorigenic potentialities of reoviruses. A variety of physical and chemical agents, including hormones, will have to be considered as possibly important supplemental factors.[29]

The results which we have obtained so far do bear on the important problem of fundamental properties which characterize the genetic material of tumor viruses. 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. First, their nucleic acid must have the required stability to assure the presence in the infected cells of viral genetic material for a considerable period of time. Continued presence of virus-specific elements has been demonstrated in all virus-induced tumors save polyoma, in which case all attempts to prove their continued presence have failed.[30-34] Second, the nucleic acid of tumor viruses must be able to replicate and to express its gene functions in ways which will allow synthesis of

host cell materials to continue, and, in addition, tumor virus genetic material must be potentially capable of subverting the cell from the path of normal growth and division to uncontrolled growth and division.[27, 34]

The DNA of tumor-producing viruses has been shown to be double-stranded in all cases,[35-39] and thus stable to thermal denaturation. The double-stranded helical configuration not only offers stability to denaturating agents, but also allows the intercellular transfer in one molecule of a relatively large amount of genetic information which may be a necessary requirement for tumor induction.

That viruses with single-stranded RNA have not been shown to be able to cause neoplastic transformation of cells may be highly significant. There is evidence that neither RNA nor DNA, when free and single-stranded, possesses the necessary stability to function as a repository of information for a prolonged period at 37°. 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 hr for single-stranded RNA, and 10 days for single-stranded DNA.[40] 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. As we have shown, the double-stranded RNA of reo- and wound tumor viruses possesses extraordinary stability to heat.

In addition to the double-stranded helical configuration, the nucleic acids of the DNA-containing tumor viruses possess an additional stabilizing characteristic manifested by the remarkable renaturation behavior of these molecules. When these nucleic acids have been denatured by heat, they have either retained full infectivity in the instances where an assay was available, or they have regained partially or completely their original hypochromicity or their original density in cesium chloride or sulfate.[31, 35, 36, 41-43] Other DNA's acquire similar reannealing capacity through treatment with ultraviolet light, X-rays, or nitrogen mustards—all tumor-inciting principles.[44, 45] The RNA's of wound tumor virus and reovirus regain their original hypochromicity upon slow cooling from 95°C. Thus, the genetic material of both viruses has the stability which we would expect in tumor viruses, and wound tumor virus is in fact tumorigenic in a susceptible host, the sweet clover plant.

Reovirus types 1 and 2 have not been demonstrated to cause any overt illness in nature; only specially adapted strains have produced illness in mice in the laboratory.[6] Reovirus 3, when inoculated in large amounts into newborn laboratory mice or hamsters, can cause an hepatoencephalomyelitis. However, animals only a few days old are no longer susceptible. It is not known in which cells of the numerous and various hosts this group of viruses preferentially multiplies and what the inapparent cytopathology may be. Under artificial conditions in tissue culture, when mouse fibroblasts are inoculated with a large amount of reovirus type 3, the effects of reovirus infection on cellular macromolecular syntheses and association of reovirus with certain cellular structures are known. Reovirus, unlike several of the cytocidal RNA viruses, does not grossly affect the synthesis of RNA and proteins in infected cells, but does inhibit DNA metabolism,[46] suggesting a specific effect on the genetic apparatus of the cell. Furthermore, in reovirus-infected cells undergoing mitosis, virus seems to be located in a preferred but not necessary association with the mitotic spindle of the host cells.[47, 48] It is possible that the

virus on the spindle could interfere with distribution of cellular genetic material during division.

Reoviruses thus have some of the necessary attributes of tumor viruses. Only further experiments can show whether they are in fact capable of causing tumors.

Summary.—The genetic material of wound tumor virus, like that of reovirus, is a large double-helical ribonucleic acid. In wound tumor virus RNA the mole per cent of guanine is 19, adenine 31, cytosine 19, and uracil 31. The melting temperature of 90°, about 3° lower than that of reovirus RNA in the same medium, probably reflects the lower G + C content in wound tumor virus RNA, namely 38 versus 44 per cent. There is no serological relationship between wound tumor virus and any of the three prototype strains of reovirus. Reoviruses possess some of the necessary attributes of viruses with tumorigenic capacity.

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*EVOLUTION OF HEMOGLOBIN IN PRIMATES**,†

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Several proposals have been made to describe the evolutionary relationships among the genes that control the structure of the polypeptide chains in the normal human hemoglobins.[1-3] At present, the best means for evaluating these proposals is by analysis of the sequence of amino acids in the polypeptide chains of hemoglobins from several vertebrate and invertebrate forms. With this information, homologies of chain structure as shown by correspondence between sequences of amino acids in entire chains, or portions of chains, help to establish the order or trend of evolution of the hemoglobin genes. Because a vast number of animals are available for this purpose, it is important that a careful choice of subjects for study be made. The principal basis for such choice should be the comparative anatomy and paleontological record of the animal groups. Thus, one will study hemoglobins from those animals that, in contemporary theory, represent stages in the development from primitive to more advanced forms, and finally to man. For this reason we have chosen to study the hemoglobins from a single mammalian order, the Primates. This order contains not only man, of whose hemoglobins so