THE INHERITANCE OF THE PROTEIN AND NUCLEIC ACID CHARACTERISTICS OF T2 AND T6 BACTERIOPHAGE*

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In the course of this past decade it has been repeatedly suggested that the four bases of the deoxyribonucleic acids function as the symbols of the genetic code of organisms (1). During this time it has also been demonstrated that the nucleic acids of various organisms not only contain the usual four bases, adenine, guanine, cytosine and thymine, but their derivatives as well. For example, the nucleic acids of some plants contain a considerable amount of cytosine as 5-methylcytosine (2, 3), while those of some bacteria harbor a small quantity of the methylated adenine, 6-methylaminopurine (4). Certain bacterial viruses contain even more unusual nucleic acids. Thymine is replaced by 5-hydroxymethyluracil in the nucleic acid of *Bacillus subtilis* phage (5), whereas cytosine occurs as its 5-hydroxymethyl derivative in the nucleic acids of viruses of the T2–C16 species (6, 7). Since hydroxymethylcytosine may occur either unsubstituted or combined with one or two molecules of glucose (7, 8), the nucleic acids of the latter phages contain five or six chemically different nucleotides.

In order to learn something of the biological function of these unusual nucleotides, a study was initiated in our laboratory several years ago the purpose of which was to determine whether the content of mono- and diglucosylated hydroxymethylcytosine in viral nucleic acid could be related to the other viral characteristics. The wild type T2 and T6 bacteriophages, which have differently glucosylated nucleic acids and have serologically distinct proteins, were therefore crossed and the inheritance of their differentiating traits was investigated. Three characteristics of these phages,—the glucose content, the host specificity and the efficiency of plating,—were found to be heritable and readily exchangeable in crosses. Because of this, it was concluded that these traits are determined genetically and that the genes which control them are not closely linked (9).

In the study which will now be presented we have endeavored to determine

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the manner in which the protein constituents of T2 and T6 phages are inherited, and to establish whether their immunological properties are related to the chemical characteristics of the two viral nucleic acids. To achieve this goal twelve phage strains were selected from the progeny of T2 x T6 crosses and the serological specificity of their head and tail proteins, as well as the extent of glucosylation of their nucleic acids, were determined. It will be seen that the majority of these viruses are hybrids which contain certain protein constituents of one parent and others of the second. Evidently, the genetic determinants of the serological specificity of viral proteins are frequently exchanged upon crossing T2 and T6. The nucleic acids of the hybrids, however, contain essentially the same quantities of the unsubstituted, mono-, and diglucosylated hydroxymethylcytosine nucleotides as does either the T2 or T6 nucleic acid. Thus, the genetic determinants of the glucosylation of viral nucleic acids recombine but rarely upon crossing the two phages. Furthermore it will be shown that the parental and hybrid viruses having similarly glucosylated nucleic acids can contain serologically different proteins, and hence the inference is warranted that there is no close linkage between the genes controlling the immunological properties of the protein constituents and those determining the extent of glucosylation of the nucleic acids of T2 and T6 bacteriophages.

Materials and Methods

Bacteriophages and Nucleic Acids.—The properties of the wild type strains of T2 and T6 phages and their hybrids used in this study were described in an earlier communication (9). Large cultures of these phages were grown on *Escherichia coli* B and purified by filtration and differential centrifugation (9). The nucleic acids of the various phages were prepared as previously reported (8).

Antisera.—The antiserum to T2 phage, No. 65, was obtained by injecting rabbits intravenously on alternate days over a period of 2 weeks with graded doses of purified T2 phage, containing 10^9 to 10^{12} particles per dose. The antisera to T2 and T6 phages, Nos. 86 and 23, respectively, were prepared similarly by injecting rabbits over a period of a month with increasing quantities of ruptured T2 or T6 phage (10^{10} to 3×10^{12} particles per dose). The latter were prepared by freezing and thawing a suspension of purified phage in buffered saline and by digesting the homogenates with 1 μg/ml of crystalline deoxyribonuclease to destroy viral nucleic acids (10). In all instances two courses of injections were given and the rabbits were bled by cardiac puncture a week after the last injection.

The antisera to the head membranes of the viruses were prepared by injecting rabbits intravenously on alternate days over a 2 week interval with graded doses of T2 and T6 doughnuts, containing 10^{10} to 5×10^{11} particles per dose. To prepare the latter, *E. coli* B cultures were infected with phage at a multiplicity of 5 P/B and the formation of virus was inhibited with proflavine (11). Infected cultures were aerated for 2 hours at 37°C, killed with chloroform, and filtered through a layer of hyflo-supercel. The filtrate was concentrated in a refrigerated Sharples centrifuge, the concentrate spun at low speed to remove bacterial debris, and the phage membranes were sedimented for 2 hours at 30,000 *g*. The membranes were then suspended in 5 ml saline and the suspension was absorbed with washed *E. coli* B cells to remove infectious phage and viral tails. After removing the bacteria, the supernatant contained approximately 5×10^{11} phage membranes and 10^7 phage particles per ml.

In order to remove cross-reacting antibodies, T2 antisera were absorbed with T6 virus, and T6 antisera with the T2. An aliquot of antiserum was mixed with an equal volume of phage suspension (2×10^{13} phage/ml), the mixture incubated 30 minutes at 37°C, and stored over night at 4°C. The precipitate was removed by centrifugation and the supernatant assayed for viral agglutinins. The absorption was repeated until the antiserum failed to agglutinate additional virus. The excess of phage was removed by absorption with heat killed *E. coli* B (2×10^9 B/ml), and the serum was diluted with saline to one-fifth of its original concentration. In order to inactivate the residual phage, the antiserum was finally heated for 30 minutes at 56°C, or allowed to stand in refrigerator for several weeks. The antisera to T2 or T6 head membranes were absorbed with phage and *E. coli* B in a similar manner.

Serological Tests.—

Agglutination tests of the various phages in the cross-absorbed T2 and T6 antisera were performed as follows. The antiserum was diluted in saline in twofold increments. The suspension of the purified phage to be tested was diluted in the same medium so as to contain 10^{11} phage/ml. 2-ml portions of the latter were then distributed into tubes and the turbidity of each determined in a photoelectric turbidimeter (12). At zero time, 0.5 ml portions of each dilution of antiserum was added to the appropriate tube, the contents mixed and the turbidity read after 30 minutes. The highest dilution of antiserum still giving a perceptible increase in the turbidity of the phage solution (2 to 5 galvanometer divisions) was considered to be the end point. The reciprocal of this dilution is termed the agglutination titer of the antiserum for a given phage.

Complement fixation tests of the various phages in antisera were performed by the photometric technique of Lanni (13). The virus was diluted in buffered saline to contain 10^{10} phage/ml, and the antiserum was diluted serially in increments of two. 0.2 ml volumes of phage, antiserum, and guinea pig complement (two 50 per cent units) were then mixed and incubated for 1 hour at 37°C. 0.4 ml of 2.5 per cent suspension of sensitized sheep erythrocytes was now added to each tube, the mixtures incubated for 1 hour at 37°C, then diluted with saline to 3.0 ml, and centrifuged to remove erythrocytes and ghosts. The absorbance of the supernatants was measured at 540 mμ and from this was calculated the highest dilution of antiserum capable of binding one unit of complement. The reciprocal of this dilution is defined as the complement fixation titer of the antiserum for the phage tested.

Neutralization tests of the phages were carried out in the following manner: The virus was diluted in nutrient broth so as to contain 4×10^5 phage/ml. The antiserum was diluted serially in increments of three. 1 ml phage was added to an equal volume of each antiserum dilution, the mixtures were incubated for 30 minutes at 37°C, and assayed on *E. coli* B. Equal volumes of phage and broth, treated in the same manner, served as control. After counting the plaques, the percentage of the surviving phage at various dilutions of antiserum was determined. From this the neutralization constants (K/minute) of the phage at each of the antiserum dilutions, which inactivated from 10 to 95 per cent of the viral particles, were calculated (14). The neutralization constants presented in the tables are the averages of several such values. In those instances in which there was no neutralization the constants were designated as zero.

Chemical Methods.—The chemical properties of the various phages and their nucleic acids were determined as previously described (8, 9). The content of the glucosylated and unsubstituted hydroxymethylcytosine derivatives in the nucleic acids was determined by the procedure of Lehman (15). The enzyme used to degrade the nucleic acids, coli-phosphodiesterase, was isolated from *E. coli* B and purified by precipitation with protamine and ammonium sulfate. The fraction precipitated at 16 per cent saturation was used for digestion (16).

EXPERIMENTAL

Serological Reactions of T2 and T6 Bacteriophages.—One of the objectives of this study was to learn the manner in which the protein constituents of the wild type strains of T2 and T6 bacteriophages are inherited. For this purpose it was necessary to characterize the protein constituents of a number of T2 x T6 hybrids and to compare them with those of the parental phages. The structure of the T-even phages is complex however, for they contain three immunologically different proteins on their surface. One is a component of the phage head membrane, the other two are constituents of the tail sheath and the tail fibers (13, 17–19). The phages also contain an internal protein which is immunologically distinct from their surface components (10). Furthermore, it has been shown that the tail constituents of T2 and T4 phages are serologically different, and that they can be interchanged upon crossing the two viruses (19, 20). Because of this, it was expected that the T2 x T6 hybrids would also contain the protein constituents of both parents the specificity of which could be determined by immunological methods.

Before undertaking this study it was necessary to select appropriate serological reactions to identify the components of one parental phage in the presence of those of the other. For this purpose the antisera to T2 and T6 phages and their membranes were chosen for study. Since the antiphage sera contain both antibodies to the homologous and the heterologous phage (21), the latter were first removed by absorption. To characterize the cross-absorbed antisera, their agglutination, complement fixation, and neutralization reactions with T2 and T6 phages were studied. It is known that the head and tail constituents of phages participate in the first two tests, but only the tail components, the sheaths and fibers, react in the neutralization test (13, 19, 22).

These experiments are summarized in Table I, where it is seen that the cross-absorbed antisera to T2 phage (Nos. 65 and 86) or to T6 virus (No. 23) agglutinated and neutralized the homologous phage at high dilution and fixed complement as well. These antisera failed, however, to react with the heterologous phage, or at best reacted but weakly. It is apparent that the cross-absorbed antiphage sera contained a large quantity of antibodies to the homologous phage, but only a small amount of antibodies reacting with the heterologous virus. The cross-absorbed antisera to the head membranes of T2 and T6 phages also reacted only with the corresponding homologous virus. As one might expect, these antisera agglutinated and fixed complement with the homologous virus at high dilution, yet neutralized the phage but slowly. Thus antimembrane sera contained a considerable quantity of antibodies reacting with head constituents of the homologous phage and only a small amount directed against the tail components. Finally, it should be stated that after absorption with homologous phage, the antisera to ruptured T2 and T6 (Nos. 86 and 23) failed to react with intact phage. Each of these antisera, however,

fixed complement with the ruptured T2 and T6 phage at a 180 to 360 dilution. It is evident that the two antisera contained small amounts of antibodies directed against viral internal proteins, which do not react with the surface components of the virus.

The data indicate clearly that the cross-absorbed antisera to T2 and T6 phages contain primarily antibodies directed toward the surface constituents of the homologous phage. By studying the agglutination or complement fixation reactions of a T2 x T6 hybrid in the two antiphage sera, it should therefore be possible to learn whether the hybrid contains the surface constituents of one

TABLE I

Serological Reactions of T2 and T6 Phages in Cross-Absorbed Antiphage and Antimembrane Sera

Antiserum	T2 phage			T6 phage		
	Aggl.*	C.F.	Neutr.	Aggl.	C.F.	Neutr.
Anti-T2 serum 65 absorbed with T6	25,600	7,680	16,000	<100	<120	49
Anti-T2 serum 86 absorbed with T6	25,600	7,680	4,680	<100	<120	4
Anti-T6 serum 23 absorbed with T2	<100	<120	0	12,800	3,840	6,770
Anti-T2 membrane serum 97 absorbed with T6 phage	1,600	480	8	<50	<60	0
Anti-T6 membrane serum 35 absorbed with T2 phage	<50	<60	0	3,200	1,920	96

Aggl., agglutination titer; C.F., complement fixation titer; Neutr., neutralization constant (K/minute).

or both parental phages. Furthermore, since the viruses are neutralized only by the antibodies to the viral tail constituents, the study of neutralization reactions of a hybrid in the two antiphage sera should reveal whether the tail sheaths and fibers of the latter have the serological specificity of the same parent, or whether one of these components is derived from T2 phage and the other from T6. Finally, since the T2 and T6 membrane antisera contain mainly the antibodies to viral head protein, the serological specificity of the head membranes of a hybrid can be unequivocally determined by performing its agglutination or complement fixation tests in these two sera.

The Serological Specificity of Phages Derived from T2 x T6 Crosses.—When two genetically related phages multiply in the same bacterial cell, segments of their genomes may be exchanged and as a result hybrids arise in the progeny. Our earlier study concerning the inheritance of host specificity, efficiency of plating, and glucose content of T2 and T6 phages revealed that upon crossing the two viruses six types of phages are formed. Two had the characteristics of parental phages, whereas the remaining four were hybrids (9). To learn

whether the immunological properties of these phages would also differ, agglutination tests of thirty-seven viral strains described in the previous communication (9) were performed in the cross-absorbed T2 and T6 antisera. These preliminary tests revealed that twenty-two strains were agglutinated by both T2 and T6 antisera, six reacted only with the former serum and nine with the latter. Thus, the crossing of T2 with T6 resulted in progeny which acquired the serological properties of both parents.

TABLE II

Characteristics of Phage Strains Derived from Crosses between T2 and T6

Phage strain No.	Host range*	Efficiency of plating‡	Glucose content§	Genotype of the original strain‖
1	T2	0.23	12.2	*h2 ef2 g2* (1)
2	T2	0.11	11.4	" (21)
3	T2	0.86	11.5	*h2 ef6 g2* (22)
4	T2	0.80	12.4	" (7)
5	T2	1.33	27.0	*h2 ef6 g6* (29)
6	T2	1.18	25.6	" (9)
7	T6	1.04	25.6	*h6 ef6 g6* (35)
8	T6	1.01	25.5	" (17)
9	T6	1.31	12.3	*h6 ef6 g2* (15)
10	T6	1.27	12.9	" (16)
11	T6	0.04	12.4	*h6 ef2 g2* (12)
12	T6	0.09	13.6	" (10)

* Determined by plating the phage on *E. coli* strains B, B/2, and B/6.

‡ The ratio of phage titers on *E. coli* K12 and *E. coli* B.

§ Moles glucose per 100 moles phosphorus.

‖ Symbols *h2*, *ef2*, and *g2* indicate that genes controlling the host range, efficiency of plating and glucose content of the virus are those of T2; *h6*, *ef6*, and *g6* denote the corresponding genes of T6. The figures in parenthesis are serial numbers used to designate the viral strains in the previous communication (9).

In order to study the protein constituents of these hybrids, and to determine whether a relationship exists between the various traits of T2 and T6, twelve strains, two of each of the six phage types described above, were chosen for further investigation. The phages were so selected that one strain of each pair was agglutinated by both T2 and T6 antisera, whereas the other reacted only with one. Phage stocks were prepared from single plaques of each strain and large viral cultures were grown. The phages were then isolated, purified and their properties were examined.

The biological and chemical characteristics of the twelve viruses were determined first. The results are listed in Table II. As can be seen, strains 1 and 2 resembled T2 phage; for both had the host specificity of T2, plated with a low

efficiency on *E. coli* K12, and contained 11 to 13 moles of glucose per 100 moles phosphorus. The properties of strains 7 and 8 were similar to those of T6 virus. The host range of the two phages was the same as that of T6, their efficiency of plating on the K12 bacillus was high, and they both contained some 26 moles of glucose per 100 moles phosphorus. The remaining eight strains were hybrids and they differed from the parental phages by at least one of their traits. Moreover, it can be seen in the table that the traits of each of the twelve strains

TABLE III

Agglutination and Complement Fixation Reactions of Various Bacteriophages in Cross-Absorbed Antiphage Sera

Phage strain No.	Genotype	Agglutination* in antiserum		Complement fixation‡ in antiserum		Specificity of phage antigens§
		T2-65	T6-23	T2-65	T6-23	
1	*h2 ef2 g2*	25,600	<100	3,840	<120	2
2	"	25,600	1,600	960	3,840	2,6
3	*h2 ef6 g2*	25,600	<100	3,840	<120	2
4	"	25,600	1,600	1,920	3,840	2,6
5	*h2 ef6 g6*	25,600	<100	7,680	<120	2
6	"	25,600	800	3,840	480	2,6
7	*h6 ef6 g6*	<100	12,800	<120	3,840	6
8	"	25,600	6,400	3,840	960	6,2
9	*h6 ef6 g2*	100	12,800	120	7,680	6,(2)
10	"	6,400	6,400	7,680	1,920	6,2
11	*h6 ef2 g2*	800	6,400	480	3,840	6,(2)
12	"	6,400	3,200	7,680	480	6,2

*, ‡ The figures indicate the agglutination and complement fixation titers, respectively.

§ Nos. 2 and 6 indicate that the antigens of the phage have serological specificity of T2 and T6 virus, respectively. The numbers in parenthesis indicate the specificity of the minor antigenic component of a virus.

corresponded to the genotypes of the phages from which they were derived. Thus all strains bred true, and hence their genotypes were stable.

Agglutination and complement fixation reactions of the twelve strains in the cross-absorbed T2 and T6 antisera were then studied. The results of these tests are summarized in Table III. Here it is seen that all phages having the host range of T2 (strains 1 to 6) were agglutinated in highly diluted T2 antiserum and showed differences when the tests were made in T6 antiserum. Strain 1, 3, and 5 failed to react with the latter whereas strains 2, 4, and 6 were agglutinated, although at a lower dilution than was the parental T6 (Table I). In contrast to this, the viruses having the host range of T6 (strains 7 to 12) were all agglutinated by highly diluted T6 antiserum and exhibited differ-

ences when tested in T2 antiserum. Strains 7, 9, and 11 reacted with the latter weakly or not at all, whereas strains 8, 10, and 12 were agglutinated by high dilutions of the antiserum. It is apparent therefore that strains 1, 3, and 5 contained antigens of T2, strain 7 had those of T6, and the remaining eight contained either certain antigenic constituents of T2 and others of T6, or possibly new antigens having the serological specificity of both parental phages.

The results of complement fixation tests confirmed this conclusion. In addition they revealed that strains 2, 4, 9, and 11 fixed complement with T6 antiserum more strongly than with the anti-T2 serum, and that strains 6, 8, 10, and 12 had a lower complement fixation titer in the former antiserum than in the latter. This indicates that the first four hybrids contained more antigens or antigenic determinants of T6 virus than of T2, while the ratio of the two parental antigens in the remaining four hybrids was reversed.

Thus it became evident that several types of hybrids are formed upon crossing T2 and T6. Some contain antigenic constituents of only one parental phage, whereas the others contain different proportions of the antigens or the antigenic determinants of both parents.

Serological Specificity of Head and Tail Proteins of T2 x T6 Hybrids.—Since certain T2 x T6 hybrids reacted with both T2 and T6 antisera it was necessary to determine the immunological properties of their surface constituents. The serological specificity of the head proteins of the twelve viruses was therefore ascertained by testing their agglutination in T2 and T6 membrane antisera, and that of the tail components by examining their neutralization in the two antiphage sera. The results are shown in Table IV.

It will be seen that phages having the host range of T2 (strains 1 to 6) differed greatly in their agglutinability in the cross-absorbed antimembrane sera. Strains 1, 3, 5, on the one hand, had high agglutination titers in the T2-membrane antiserum and failed to react with the anti-T6-membrane serum. Strains 2 and 4, on the other hand, reacted but moderately in the former and were agglutinated strongly by the latter, while strain 6 was agglutinated at the same dilution of both antisera. It is apparent that strains 1, 3, and 5 contained head antigens of T2 phage, whereas strains 2, 4, and 6 had head membranes having the serological specificity of both parents. The membranes of strains 2 and 4, which exhibited higher agglutination titers in T6-membrane sera than did strain 6, apparently contained more antigens or antigenic groupings of T6 virus and less of T2, than the membranes of the latter strain. The neutralization tests revealed that all six phages were inactivated rapidly by the antiserum to T2 phage and did not react significantly with anti-T6 serum. These observations indicate that strains 1 to 6 contained the tail sheaths and fibers of T2 virus.

The phages having the host range of T6 (strains 7 to 12) also differed in their serological reactions. Thus, strains 7, 9, and 11 were agglutinated only

by the antiserum to T6 membranes, and hence their head membranes contained the antigens of T6 phage. Strains 8, 10, and 12, on the other hand, reacted with both antimembrane sera, and consequently they contained the membranes having the serological specificity of both parental viruses. The neutralization tests revealed moreover that the six strains were inactivated rapidly by T6 antiserum and reacted only moderately with anti-T2 serum. Since the rates of neutralization of hybrids 7 and 12 in both antisera were comparable to those

TABLE IV

Serological Reactions of Various Phages in Cross-Absorbed Antiphage and Antimembrane Sera

Phage strain No.	Genotype	Agglutination* in antimembrane serum		Neutralization‡ in antiphage serum		Specificity of phage proteins§		
		T2M-97	T6M-35	T2-65	T6-23	Head	Sheath	Fibers
1	*h2 ef2 g2*	800	<50	12,100	0	2	2	2
2	"	200	1,600	25,000	5	2,6	2	2
3	*h2 ef6 g2*	800	<50	25,300	0	2	2	2
4	"	200	1,600	28,100	8	2,6	2	2
5	*h2 ef6 g6*	800	<50	25,000	9	2	2	2
6	"	800	800	16,200	1	2,6	2	2
7	*h6 ef6 g6*	<100	3,200	81	8,290	6	6	6
8	"	800	1,600	294	4,230	6,2	2	6
9	*h6 ef6 g2*	<100	1,600	140	2,390	6	2	6
10	"	800	800	380	2,100	6,2	2	6
11	*h6 ef2 g2*	<100	1,600	420	8,150	6	2	6
12	"	800	800	65	3,260	6,2	6	6

* Agglutination titer.

‡ Neutralization constant (K/minute).

§ Figures 2 and 6 indicate that the phage protein in question has the serological specificity of the corresponding constituent of T2 and T6 phage, respectively. Symbols 2,6 or 6,2 indicate that the constituent has the specificity of both parental phages.

of T6 virus (Table I) it was concluded that these hybrids contained the tail sheaths and fibers of T6 phage. Strains 8 to 11 also contained a major tail component having the serological specificity of T6 phage, for they all reacted rapidly with T6 antiserum. However, since these four were neutralized by T2 antiserum faster than T6 phage, there was an indication that these phages might contain an additional tail constituent having the T2 specificity.

Others have shown that the crossing of T2 with T4 yields a hybrid which contains the tail sheath of T2 and the tail fibers of T4. This hybrid has the host range of T4 virus and is neutralized by T4 antiserum absorbed with T2 phage as rapidly as is the parental T4. Furthermore it cross-reacts with

the T2-antiserum absorbed with T4 approximately seven times faster than T4 virus (19). Since hybrids 8 to 11, having the host range of T6, are neutralized rapidly by the cross-absorbed T6 antiserum and react moderately with the T2 antiserum, it is justifiable to conclude that each contains the tail fibers of T6 virus and the sheaths of T2.

The serological specificity of the surface components of each of the twelve strains is listed in the last column of Table IV, where it is seen that two-thirds of the hybrids contain constituents of both parental phages. It is evident that upon crossing T2 with T6 their surface components are frequently interchanged. During this process the serological specificity of the tail fibers of the parental phages remains unaltered, or is modified but slightly, for all hybrids are neutralized rapidly only by one of the antisera. The specificity of the tail sheaths also seems to be unchanged on crossing, for hybrids which cross-react with the second antiserum are all neutralized at similar rates. In contrast to this the serological specificity of the head membranes of parental phages undergoes a considerable alteration on crossing, for some hybrids are agglutinated by the T2 and T6 membrane antisera equally well whereas others react strongly with one of the antisera and weakly with the other. Whether such membranes are composed of a mosaic of T2 and T6 protein molecules, or consist of identical molecules having the antigenic groupings of both parental phages, is not yet known.

The Inheritance of the Immunological Properties of T2 x T6 Hybrids.—To learn whether the serological properties of the twelve hybrids are heritable, single plaque subcultures of each were prepared and large samples grown. It was ascertained that each subculture had the host range, efficiency of plating and glucose content of the strain from which it was derived. The agglutination reactions of each in antimembrane sera and their neutralization reactions in antiphage sera were then investigated. T2 antiserum 86 was used in the neutralization tests because the antibodies which cross-react with T6 phage could be more satisfactorily removed than from serum 65. These experiments are summarized in Table V.

It can be seen that the agglutination titers of the twelve subcultures in the T2 and T6 antimembrane sera were similar to those of the phage strain from which they were derived (Table IV). This fact indicates that the head membranes of each subculture and those of the corresponding original strain had essentially the same serological specificities. The neutralization tests revealed that all subcultures reacted with the antiphage sera essentially in the same manner as the original strains. Subcultures 1 to 6 were neutralized rapidly with the T2 antiserum and failed to react with the T6 antiserum, whereas subcultures 7 to 12 were neutralized rapidly by the T6 antiserum and differed in their reactivity with the T2 antiserum. In the latter instance the subcultures of strains 7 and 12 reacted very slowly, whereas those of strains 8 to 11 were

neutralized at moderate rates. It is therefore apparent that subcultures 1 to 6 contain tail components of T2 phage, those of strains 7 and 12 the tail components of T6 phage, and those of strains 8 to 11 the tail sheaths of T2 phage and the tail fibers of T6.

It is evident that the serological specificities of the head membranes, tail sheaths, and tail fibers of each subculture were essentially the same as those

TABLE V

Serological Reactions of Single Plaque Subcultures of Various Phages in Cross-Absorbed Antiphage and Antimembrane Sera

Original strain		Subcultures				
Phage strain No.	Genotype	Agglutination* in antimembrane serum		Neutralization‡ in antiphage serum		Genetic determinants of protein specificity§
		T2M-97	T6M-35	T2-86	T6-23	
1	*h2 ef2 g2*	1,600	<50	5,550	0	*m'2 m"2 s2 f2*
2	"	200	3,200	8,200	2	*m'2 m"6 s2 f2*
3	*h2 ef6 g2*	1,600	<50	5,710	0	*m'2 m"2 s2 f2*
4	"	100	1,600	5,500	0	*m'2 m"6 s2 f2*
5	*h2 ef6 g6*	800	<50	8,750	0	*m'2 m"2 s2 f2*
6	"	1,600	800	5,560	2	*m'2 m"6 s2 f2*
7	*h6 ef6 g6*	<50	3,200	3	7,440	*m'6 m"6 s6 f6*
8	"	800	1,600	384	3,260	*m'6 m"2 s2 f6*
9	*h6 ef6 g2*	<50	1,600	218	3,210	*m'6 m"6 s2 f6*
10	"	800	800	505	4,200	*m'6 m"2 s2 f6*
11	*h6 ef2 g2*	<50	1,600	629	8,300	*m'6 m"6 s2 f6*
12	"	800	800	2	3,650	*m'6 m"2 s6 f6*

* Agglutination titer.

‡ Neutralization constant (K/minute).

§ Symbols *m'2* and *m"2*, *s2*, and *f2* designate genes controlling the serological specificity of the head membranes, tail sheaths and tail fibers of T2 phage, respectively. *m'6* and *m"6*, *s6*, and *f6* denote the corresponding genes of T6 virus.

of the strains from which they were derived. Since each originated from a single plaque, it can be concluded that the serological properties of each viral strain are transmitted by a single phage particle and that the serological specificity of each of the surface components of hybrids is a heritable trait.

The Genetics of the Protein Constituents of T2 and T6 Phages.—The genetic determinants which control the immunological characteristics of the head and tail proteins of T2 and T6 phages have different properties. As can be seen in Tables IV and V, hybrids which are agglutinated by both T2 and T6 antimembrane sera occur quite frequently, a fact which indicates that the immunological properties of the head proteins of the two parental phages are readily

blended upon crossing. It is therefore necessary to conclude that the serological specificity of the head membranes of T2 or T6 phage is controlled by at least two distantly linked recombining genes which can be designated by symbols *m'2*, *m"2* and *m'6*, *m"6*, respectively. On the other hand, the hybrids having a host range of T2 were found to be neutralized by the anti-T2 sera at rates similar to that of T2 phage, while those having the host specificity of T6 were inactivated by T6 antiserum as rapidly as was T6 virus. It has been shown by others that the host specificity of a phage and its susceptibility to neutralization by a specific antiserum are functions of its tail fibers (19). It is apparent therefore that all T2 x T6 hybrids contained tail fibers having essentially the same properties as those of T2 or T6. This fact indicates that the serological specificity of the fibers of parental phages is not changed upon crossing, and hence, it can be inferred that the immunological properties of the tail fibers of T2 and T6 may be controlled by single allelic genes, *f2* and *f6*. It can also be seen in the tables that hybrids 8 to 11, which had tail fibers of T6, were neutralized not only by T6 antiserum but also by anti-T2 sera. Since a T2 x T4 hybrid having sheaths of one parent and fibers of the other was found to be inactivated by antisera to both parental viruses (19), it was assumed that the four hybrids in question contain the tail sheaths having the specificity of T2. It would seem therefore that the serological specificity of the tail sheaths of T2 and T6 phages may also be controlled by single genes, which will be termed *s2* and *s6*.

The genetic determinants controlling the immunological specificity of the surface proteins of each hybrid are listed in the last column of Table V. A comparison of the genotypes reveals that half of the hybrids contained *m'* gene of one parent and the *m"* of the other. It would appear, therefore, that the *m'* and *m"* genes of T2 or those of T6 phage are indeed distantly linked. Since the proportions of T2 and T6 antigens in the head membranes of hybrids may vary, it is possible that the action of the *m'* or *m"* genes may be controlled by a modifier. The *s* and *f* genes of the two parental phages, and their *s* and *m* determinants also, are not closely linked, for hybrids which have the *s2 f6* or *m'6 m"6 s2* genotypes are encountered in the progeny. In contrast to this, the linkage between the *f* and one of the *m* genes of T2 and T6 viruses may be close, for *f2* gene is associated with *m'2* and the *f6* is accompanied by *m'6*.

Finally, if one examines the interrelation of the genes controlling the specificity of the protein constituents of hybrids and those governing their host range, efficiency of plating, and glucose content, (the *h*, *ef*, and *g* genes, Table V) it can be seen that the *h2* determinant is always associated with *f2*, and that the *h6* gene is accompanied by *f6*. This is not surprising, for it is known that the host specificity of T2 and T4 phages and their ability to be neutralized by homologous antisera are properties of the viral tail fibers (19, 20). It is therefore possible that the genes controlling these two traits are identical, or

at least very closely linked. In contrast to this there is no correlation between the *g* and *ef* genes of T2 and T6 phages, and their *m'*, *m"*, *s*, and *f* determinants, for the hybrids may contain the *g* or *ef* genes of one parent and any one of the four protein determinants of the other. It is necessary therefore to conclude that no close linkage exists between the genetic determinants controlling the glucose content or the efficiency of plating of T2 or T6 phages and the determinants which govern the serological specificity of the protein constituents of these viruses.

The Inheritance of the Chemical Characteristics of the Nucleic Acids of T2 and T6 Phages.—From the foregoing there appears to be no correlation between the serological specificity of the protein components of the T2 x T6 hybrids

TABLE VI

Chemical Properties of Nucleic Acids of Various Bacteriophages

Nucleic acid from strain No.	Nitrogen	Phosphorus	Glucose	Moles Glucose per 100 moles P	Genotype of phage strain*
	per cent	*per cent*	*per cent*		
5	13.0	8.0	12.8	27.5	*g6 m'2 m"2 s2 f2*
6	12.9	7.8	11.9	26.2	*g6 m'2 m"6 s2 f2*
9	14.2	8.5	6.5	13.1	*g2 m'6 m"6 s2 f6*
10	13.8	8.3	6.8	14.1	*g2 m'6 m"2 s2 f6*
T2	14.1	8.4	6.5	13.3	*g2 m'2 m"2 s2 f2*
T6	13.1	7.9	12.8	27.9	*g6 m'6 m"6 s6 f6*

* For explanation of symbols see Tables II and V.

and their glucose content. Since the hexose is a component of the nucleic acids of T2 and T6 phage (9) it would also seem that the extent of glucosylation of their nucleic acids has no effect whatsoever upon the nature of the viral proteins. It is known however, that T2 and T6 nucleic acids contain different and characteristic quantities of unsubstituted, mono-, and diglucosylated hydroxymethylcytosine (8, 15). It is possible therefore, that nucleic acids of hybrids, which have the same glucose content as T2 or T6 phage, may contain different proportions of the three hydroxymethylcytosine derivatives than those found in nucleic acids of the parental viruses. In order to ascertain whether this might be the case, nucleic acids of four hybrids were isolated and analyzed.

The gross chemical characteristics of the nucleic acids of these hybrids and of both parental phages are compared in Table VI. As can be seen the nucleic acids of hybrids 5 and 6 had essentially the same chemical properties as the nucleic acid of T6 virus. The protein constituents of hybrid 5 however, exhibited the serological specificity of T2, whereas the head membranes of hybrid 6 had the specificity of both parents and its tail proteins that of T2 phage (Table IV).

Similarly, the nucleic acids of hybrids 9 and 10 resembled chemically that of T2, but the hybrids themselves contained certain proteins of one parent and others of the second. It is apparent therefore, that upon crossing T2 with T6 the gross chemical composition of the nucleic acid of each parental phage was inherited as a unit character independently of viral protein constituents.

In order to compare the proportions of unsubstituted, mono-, and diglucosylated hydroxymethylcytosine nucleotides in the six nucleic acids, they were digested with deoxyribonuclease and coli-phosphodiesterase. The nucleotides were isolated chromatographically and the amount of each of the hydroxymethylcytosine derivatives determined (15). Since preparations of the diesterase contained small amounts of monophosphatases only 70 to 90 per cent of the total hydroxymethylcytosine was recovered as mononucleotides. The results of these experiments are summarized in Table VII.

TABLE VII

Proportions of Hydroxymethylcytosine Nucleotides in Nucleic Acids of Various Bacteriophages

Nucleic acid from strain No.	Diglucosyl-dHMP	Glucosyl-dHMP	dHMP*	Recovery‡	Genotype of phage strain§
	per cent‖	*per cent*‖	*per cent*‖		
5	72.0	4.1	23.9	90	*g6 m'2 m"2 s2 f2*
6	67.1	5.4	27.5	70	*g6 m'2 m"6 s2 f2*
9	3.5	73.6	22.9	78	*g2 m'6 m"6 s2 f6*
10	4.0	71.7	24.3	87	*g2 m'6 m"2 s2 f6*
T2	5.5	66.2	28.4	81	*g2 m'2 m"2 s2 f2*
T6	70.7	3.9	25.5	80	*g6 m'6 m"6 s6 f6*

* Hydroxymethylcytidylic acid (dHMP).

‡ Per cent of total HMC present in phage nucleic acid. The content of HMC in all T-even phages is assumed to be 17.0 moles per 100 moles P (6, 15).

§ For explanation of symbols see Tables II and V.

‖ Per cent of recovered hydroxymethylcytosine (HMC) nucleotides.

It can be seen that the proportions of the three hydroxymethylcytosine derivatives in the nucleic acids of hybrids 5 and 6 resembled those of the nucleic acid of T6 phage. The three nucleic acids contained 67 to 72 per cent of the hydroxymethylcytidylic acid diglucosylated, 4 to 5 per cent of the acid monoglucosylated, and the remainder was unsubstituted. On the other hand, the nucleic acids of hybrids 9 and 10 were similar to that of T2 virus. In these only 3 to 5 per cent of hydroxymethylcytidylic acid was combined with two molecules of glucose and some 66 to 74 per cent was linked with one molecule of the hexose.

The data indicate clearly that the proportions of the three hydroxymethylcytosine derivatives in nucleic acids of the hybrids did not deviate significantly from those found in T2 or T6 nucleic acids. Since the hybrids themselves

differed from the parental phages by a number of traits, it is apparent that the glucosylation patterns of nucleic acids of T2 and T6 phages were inherited independently of other viral characteristics. Furthermore it can be seen that the nucleic acids of hybrid 5 and T6 are predominantly diglucosylated, whereas those of hybrid 9 and T2 are monoglucosylated. The protein constituents of hybrid 5 and T6 are serologically different as are those of hybrid 9 and T2 (Table IV). It is evident therefore that the extent of glucosylation of the hydroxymethylcytosine component of viral nucleic acids is not correlated with the serological specificity of viral proteins.

DISCUSSION

The study of the inheritance of the immunological properties of bacterial viruses was initiated by Adams. He observed that upon crossing T5 and PB phages hybrids were formed which could be neutralized or could bind complement with an antiserum to either parent (22, 23). Others have subsequently demonstrated two serologically distinct components in the tail of T5 or PB phage which participate in the neutralization reaction of these viruses. It has been suggested that when the genetic determinants of the tail constituents of the two phages are interchanged hybrids arise which differ from either parent in serological specificity (24–26).

The tails of T2 and T4 phages also were found to contain two serologically different protein components, fibers and sheaths (18, 19). Both of these constituents participate in the neutralization of the virus by specific antiserum. In addition, the tail fibers react with viral receptors of bacterial cell and thus determine the host specificity of the phage. The inheritance of the characteristics of the two tail components of each phage is controlled by single genes, and these genes can be interchanged upon crossing the two phages (19, 20).

The inheritance of the serological specificity of the head proteins of T-even phages has not yet been investigated. From the data now presented it is evident that certain hybrids derived from T2 × T6 crosses are agglutinated by both T2 and T6 membrane antisera. Thus the head membranes of these hybrids have the serological specificity of both parental phages. The structure of such membranes is not yet known however. They may consist of head proteins of both parental phages or of a new protein having antigenic groupings of both parents. Our study has also revealed that half of the viral strains derived from T2 x T6 crosses contain head membranes having the immunological properties of both parents, and that these characteristics are heritable. Hence it is concluded that the serological specificity of the head proteins of T2 and T6 phages is controlled by at least two genetic determinants, which are not closely linked. Since the proportions of the two parental antigens in the head membranes of various hybrids differ it is possible that the action of one of the genes is governed by a modifier.

As one might expect, the tail constituents of T2 and T6 phages are inherited

in the same manner as those of T2 and T4 (19, 20). Thus all hybrids were found to be neutralized rapidly by the antiserum to that parental phage which had the same host specificity as the hybrid in question. This was interpreted as an indication that the tail fibers of all hybrids have essentially the same properties as the fibers of one or the other of the parental viruses. It was also observed that certain hybrids having the host specificity of T6 were neutralized not only by T6 antiserum but also by the anti-T2 serum. Hence, it was inferred that these hybrids contained tail fibers of T6 and sheaths of T2 phage. Because of this, and since the serological characteristics of all hybrids were heritable, it is concluded that the immunological properties of the sheath and fiber proteins of T2 and T6 phages are controlled by single genetic determinants, which are not closely linked.

Our investigations have also revealed that both the glucose content and the proportions of unsubstituted, mono- and diglucosylated hydroxymethylcytosine derivatives of the nucleic acids of hybrids were similar to those of the T2 or T6 nucleic acid. This indicates that the glucosylation patterns of the nucleic acids of the two parental phages were inherited intact upon crossing. It has been shown earlier (9) that the glucose content of the T2 and T6 nucleic acids is controlled by two non-allelic genes, *g2* and *g6*. The data presented here suggest that these genes not only govern the glucose content but the proportions of the three hydroxymethylcytosine nucleotides as well. Since T2 and T6 phages induce the formation of phage specific enzymes, glucose transferases, in the host bacteria (27), it is very likely that the *g2* and *g6* genes control the extent of glucosylation of the viral nucleic acids by determining the specificity of these enzymes.

Our study has had a second objective, to learn whether the presence of different proportions of the three hydroxymethylcytosine nucleotides in the nucleic acids of T2 and T6 viruses is correlated with any other heritable traits. It has previously been shown that the glucose content of phage nucleic acids is not related to the host range of the virus nor to its efficiency of plating (9). More recently the nucleic acids of rapidly lysing variants of T2, T4, and T6 phages were found to have the same glucose content as those of the wild type viruses (28), a fact which indicates that the extent of glucosylation of these substances is not related to the r or r^+ traits. The data now presented reveal that there is no correlation between the glucose content of viral nucleic acids and the serological specificity of viral proteins. Therefore, none of the traits investigated appears to be related to the glucose content of viral nucleic acid.

Moreover the number of hexose molecules linked to hydroxymethylcytosine appears to have no effect upon the immunological properties of viral proteins, for viruses having serologically identical or very similar proteins may contain either predominantly monoglucosylated or diglucosylated nucleic acids. It has been suggested, however, that the serological specificity of a protein is determined by its chemical structure (29), and that the latter is controlled, in final

analysis, by nucleotide sequences in the segment of the deoxyribonucleic acid molecule containing the appropriate structural gene (30). It is therefore very likely that both the mono- and diglucosylated hydroxymethylcytosine components of viral nucleic acids transmit the same genetic information. This inference is supported by the recent findings concerning the growth of T-even phages in bacteria incapable of producing uridine diphosphate glucose (UDPG). The viruses grown in such microorganisms contain glucose-free nucleic acids and these new phages, when grown in certain non-deficient bacteria, give rise to normal viral progeny (31–34). Thus the presence of glucose in viral nucleic acid seems to be non-essential for the transmission of the hereditary traits of these phages. Consequently, it is necessary to conclude that the unsubstituted and the glucosylated hydroxymethylcytosine components of viral nucleic acids function as identical symbols of the genetic code of the T-even bacteriophages.

SUMMARY

A study of the immunological properties of phage strains derived from T2 x T6 crosses revealed that the majority of the progeny differ serologically from the parental viruses. Some hybrids were found to contain head membranes having the serological specificity of both T2 and T6 phages, while others contained tail sheaths of the former and the tail fibers of the latter. Since the immunological properties of all hybrids were heritable, it has been concluded that the serological specificity of the head proteins of T2 and T6 is controlled by at least two genetic determinants, and that the specificities of the fiber and sheath proteins may be governed by single genes.

Furthermore it was found that nucleic acids of hybrids had similar proportions of unsubstituted, mono- and diglucosylated hydroxymethylcytosine nucleotides to the nucleic acid of either T2 or T6 phage. Since the parental and hybrid viruses having chemically similar nucleic acids contained in some instances serologically different proteins, it has also been concluded that the extent of glucosylation of the hydroxymethylcytosine component of viral nucleic acids and the immunological properties of viral proteins are independently heritable traits of T2 and T6 bacteriophages.

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