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On the Structure of the Glucosylated Hydroxymethylcytosine Nucleotides of Coliphages T2, T4, and T6

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Although pancreatic deoxyribonuclease and snake venom phosphodiesterase quantitatively degrade many deoxyribonucleic acids to mononucleotides, the DNA of coliphages T2, T4, and T6 is resistant to complete hydrolysis. As shown by Sinsheimer (1), Volkin (2), Jesaitis (3), and Lichtenstein and Cohen (4) the hydroxymethylcytosine, which replaces cytosine in these viruses (5), is recovered in only 20 to 30% yield as the mononucleotide. The remainder, together with smaller amounts of the other three deoxyribonucleotides, is found in an enzyme-resistant polynucleotide "core." Some of the HMC[1] of these coliphages is known to be substituted with glucose and the incomplete degradation by the combined action of pancreatic DNase and snake venom phosphodiesterase has been attributed to the failure of the diesterase to attack most of the internucleotide linkages in which glucosylated HMC is involved (1–4). Cohen (6) has reported a similar resistance of T6 DNA to degradation by pancreatic DNase and a calf intestinal phosphatase preparation.

We have recently found an enzyme in *Escherichia coli* which can quantitatively degrade the DNA of these bacteriophages to 5′-mononucleotides (7) and have examined in detail all of the HMC nucleotides present in these viral DNA's. The results obtained demonstrate that the DNA's from phages T2 and T6 contain 3 distinct HMC nucleotides, bearing 2, 1, or 0 moles of glucose per mole of nucleotide. In T2 DNA, the nucleotide with a single mole of glucose is the predominant form; in T6, the HMC nucleotide containing 2 moles of glucose predominates. In DNA from T4 phage, only the monoglucosylated HMC is present.

Further examination of the isolated monoglucosylated HMC nucleotides with respect to the nature of the glycosidic linkage has shown that whereas all the glucose in the monoglucosylated HMC from T2 is linked in the α configuration, T4 contains two monoglucosylated HMC nucleotides, one with the glucose bound in the α configuration and a second in which the glucose is in the β configuration.

Since the extent of glucosylation is a "species marker" in bacteriophage genetics, these new details regarding phage DNA structure are pertinent to the interpretation of available genetic data and suggest lines of future investigation.

EXPERIMENTAL PROCEDURE

Materials and Methods

Crystalline pancreatic DNase and RNase were purchased from the Worthington Biochemical Corporation. Glucose 6-phosphate dehydrogenase (Zwischenferment) containing hexokinase was purchased from the Sigma Chemical Company. Purified human semen monoesterase was the gift of Dr. Leon A. Heppel. The DNA phosphodiesterase from *E. coli* was purified and assayed as described previously (7). Crystalline bovine plasma albumin was obtained from the Armour Laboratories. Diethylaminoethyl cellulose (DEAE-cellulose) was purchased from Brown and Company (Berlin, New Hampshire). *p*-Nitrophenyl α-D-glucopyranoside was generously provided by Dr. H. Halvorson, and *o*-nitrophenyl β-D-glucopyranoside by Dr. Melvin Cohn.

Partial Purification of α-Glucosidase—α-Glucosidase was assayed according to the spectrophotometric method of Halvorson which measures the continuous release of *p*-nitrophenol from *p*-nitrophenyl α-D-glucopyranoside (8). The reaction mixture contained, in a final volume of 1.2 ml, 67 μmoles of potassium phosphate buffer, pH 6.5, 1.3 μmoles of cysteine, 0.4 mg of substrate, and 0.25 to 2.5 units of enzyme. A unit is defined as that amount of enzyme causing an increase in optical density at 420 mμ of 0.100 per minute. This corresponds to the hydrolysis of about 0.01 μmole of substrate per minute.

The enzyme source was a lyophilized preparation of maltose-grown *Saccharomyces italicus* strain Y 1225, kindly provided by Dr. H. Halvorson. The dried cells (6.8 g) were suspended in 20 ml of 0.1 M potassium bicarbonate and incubated at 37° for 6 hours. Unless otherwise specified, all further operations were carried out at 0–4°. The suspension was centrifuged for 10 minutes at 10,000 × *g*. The supernatant fluid (20 ml) was diluted with an equal volume of 0.05 M potassium phosphate buffer, pH 6.5, and 12.8 g of ammonium sulfate were added. After 5 minutes the precipitate was removed by centrifugation for 5 minutes at 10,000 × *g*. To the supernatant fluid were added an additional 5.6 g of ammonium sulfate; the resulting precipitate was collected as before, and dissolved in 13 ml of 0.07 M potassium phosphate buffer, pH 6.5, containing 10^{-4} M 2-mercaptoethanol. RNase was added to a final concentration of 0.1 μg per ml and the solution was incubated at room temperature for 1 hour. The RNase-treated ammonium sulfate fraction was then dialyzed for 16 hours against 0.02 M potassium phosphate buffer, pH 8.0, containing 10^{-4} M 2-mercaptoethanol, and fractionated further by chromotography on DEAE-cellulose (9).

A column of DEAE-cellulose, 10 × 2.2 cm, was equilibrated with 0.02 M potassium phosphate buffer, pH 8.0; the dialyzed ammonium sulfate fraction was applied to the column and the adsorbent was washed with the same buffer. A linear gradient was applied with 0.03 and 0.15 M potassium phosphate, pH 6.5 (200 ml of each) as limiting concentrations, and the flow rate was adjusted to 60 ml per hour. The active fractions were eluted between 3 and 4 column volumes of effluent and were pooled

[1] The abbreviations used are: HMC, 5-hydroxymethylcytosine; dHMP, 5-hydroxymethyldeoxycytidine 5′-monophosphate.

(65 ml). Ammonium sulfate, 37 g, was added to the pooled fractions, and after 10 minutes the precipitate was collected by centrifugation at 12,000 × *g* for 20 minutes. The precipitate was dissolved in 2 ml of 0.05 M potassium phosphate at pH 7.0 and dialyzed for 16 hours against 0.05 M potassium phosphate buffer, pH 7.0, containing 10^{-4} M 2-mercaptoethanol. The dialyzed DEAE fraction contained 30% of the activity initially present in the extract and represented an over-all purification of about 10-fold. This preparation had no detectable β-glucosidase activity (<0.001% of its α-glucosidase activity) as measured by the hydrolysis of *o*-nitrophenyl β-D-glucopyranoside. It was also free of 5′-nucleotidase activity (<1% of its α-glucosidase activity) as indicated by its failure to release a measurable amount of *ortho* P^{32} from P^{32}-labeled 5′-dCMP. Hydrolysis of the glucosylated HMC nucleosides by the α-glucosidase preparation proceeded at about 1/100 the rate observed with the *p*-nitrophenyl α-D-glucopyranoside when measured at a comparable substrate concentration. Large differences in the rate of hydrolysis of various α-glucosides by the yeast α-glucosidase, depending upon the nature of the aglycone moiety, have been observed by Halvorson and Ellias (8).

Partial Purification of β-Glucosidase—β-Glucosidase was assayed by measuring the continuous release of *o*-nitrophenol from *o*-nitrophenyl β-D-glucopyranoside. The reaction mixture (1.2 ml) consisted of 34 μmoles of potassium phosphate buffer, pH 6.5, 12.8 mg of substrate, and 0.2 to 1.0 unit of enzyme. A unit of β-glucosidase is defined as that amount of enzyme causing an increase in optical density at 420 mμ of 0.100 per minute.

The almond β-glucosidase preparation purchased from the Nutritional Biochemicals Company contained α-glucosidase activity (about 1% of its β-glucosidase activity) as measured by the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside. This contaminating activity could be largely removed by fractionation with ammonium sulfate. To 60 ml of the β-glucosidase solution (4°), containing 600 mg of protein in 0.07 M potassium phosphate buffer, pH 6.5, were added 19 g of ammonium sulfate. After 5 minutes at 0°, the precipitate was removed by centrifugation for 5 minutes at 10,000 × *g*. To the supernatant fluid were added an additional 4.2 g of ammonium sulfate and after 5 minutes the precipitate was removed by centrifugation and dissolved in 5 ml of 0.07 M potassium phosphate buffer, pH 6.5. This solution was dialyzed for 16 hours at 4° against 0.07 M potassium phosphate buffer, pH 6.5. The dialyzed ammonium sulfate fraction was purified 3-fold over the starting preparation. It contained only barely detectable α-glucosidase activity (about 0.001% of its β-glucosidase activity) as measured by the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside.

Hydrolysis of the glucosylated HMC nucleosides by the β-glucosidase preparation proceeded at about 1/10 the rate observed with the *o*-nitrophenyl β-D-glucopyranoside, when measured at the same substrate concentration.

Growth and Purification of Bacteriophages—Bacteriophage titrations were carried out as described by Adams (10). T2r+ bacteriophage was grown and purified by the method of Herriott and Barlow (11). T4r+ and T6r+ phages were grown in the following way: 20-liter cultures of *E. coli* strain B were grown with vigorous aeration in M-9 medium (12) to a cell density of 6 × 10^8 cells per ml. L-Tryptophan to yield a final concentration of 1 μg per ml and virus (about 1 × 10^8 particles per ml of culture) were added. Aeration was continued for 7 hours after which time lysis of the cultures was complete. Titers of 2 to 3 × 10^{11} phage per ml were routinely obtained in this way. The bacterial debris was allowed to settle out at 4° and the supernatant fluid was siphoned off. The phage were then harvested by precipitation at pH 4 and purified as described by Herriott and Barlow for T2.

Since there is a problem of contamination of one preparation by another when dealing with large quantities of virus, each preparation was checked by plating on various phage-resistant mutants of *E. coli* strain B. In only one instance was a given preparation contaminated with as much as 0.01% of another phage (contamination of T4 with T2), and in all other cases the contamination was considerably less.

DNA was isolated from the purified phage as described previously (7).

Enzymatic Hydrolysis of Bacteriophage DNA's—The bacteriophage DNA's were enzymatically degraded to 5′-mononucleotides by the combined action of pancreatic DNase and the *E. coli* phosphodiesterase (7). The reaction mixture consisted of phage DNA (70 μmoles of phosphorus), 0.4 μg of pancreatic DNase, 130 μmoles of Tris buffer, pH 7.5, 13 μmoles of $MgCl_2$, and 1.3 mg of bovine plasma albumin in a final volume of 13.0 ml. After incubation at 37° for 30 minutes, the mixture was heated for 10 minutes at 80°. The pH was adjusted to about 9 with 1 N KOH and 1 mmole of glycine buffer, pH 9.2; 100 μmoles of $MgCl_2$ and 2000 units of diesterase (DEAE fraction) were added. The final volume at this point was 16 ml. Incubation was continued at 37° for 6 hours at which time 90 to 100% of the phosphate had become susceptible to semen phosphomonoesterase action. The reaction mixture was heated at 100° for 5 minutes and the precipitated protein was removed by centrifugation at 10,000 × *g* for 5 minutes. The pH was adjusted to 9.5 by the addition of 1 N NH_4OH and the digest was applied to a column of Dowex 1 (acetate form, 10% cross-linked), 15 × 1 cm. The mononucleotides were chromatographed at 4° with the use of ammonium acetate buffers at pH 4.3 according to the method of Sinsheimer and Koerner (13).

The column eluates containing the monoglucosylated 5-hydroxymethyldeoxycytidine 5′-monophosphate (monoglucosyl dHMP) were pooled and concentrated by distillation under reduced pressure, a procedure which also removed most of the ammonium acetate. The eluates containing the diglucosylated 5-hydroxymethyldeoxycytidine 5′-monophosphate (diglucosyl dHMP) and dHMP were treated in a similar manner. At pH 1, the glucosylated and nonglucosylated HMC nucleotides showed identical ultraviolet absorption spectra with a maximum at 284 mμ, aM = 13.2 × 10^3 and λ280/λ260 = 2.57. At pH 4.3, the glucosylated and nonglucosylated nucleotides were distinguished spectrophotometrically as described by Sinsheimer (1).

The various HMC nucleotides were converted to the corresponding nucleosides by treatment with semen phosphomonoesterase. The reaction mixture (0.35 ml) was composed of 0.66 μmole of HMC nucleotide, 30 μmoles of sodium acetate buffer, pH 5.0, and 135 units of semen monoesterase (an amount which produces orthophosphate from 5′-AMP at a rate of 135 μmoles per hour). The mixture was incubated at 37° for 4 hours and then heated for 2 minutes at 100°.

Glucose (measured as reducing sugar) bound to HMC nucleotide was determined by the method of Park and Johnson (14) after hydrolysis in 1 N HCl at 100° for 1 hour. Glucose released from glucosylated HMC nucleosides by the action of α- or β-glucosidases was measured by the coupled hexokinase-Zwi-

schenferment assay (15). This reaction is specific for glucose. The glucose content of intact phage DNA was estimated by the anthrone procedure (16). Correction for color produced by deoxyribose was made by subtracting the value obtained with an amount of calf thymus DNA corresponding to the concentration of phage DNA used in the analysis. Phosphate was determined by the method of Fiske and SubbaRow (17).

TABLE I

Glucose content of bacteriophage DNA's

DNA source	Molar ratios	
	Glucose-phosphate	Glucose-HMC*
T2	0.140	0.82
T4	0.178	1.05
T6	0.245	1.45

* Value calculated assuming an HMC concentration of 0.17 mole per mole of phosphate (5).

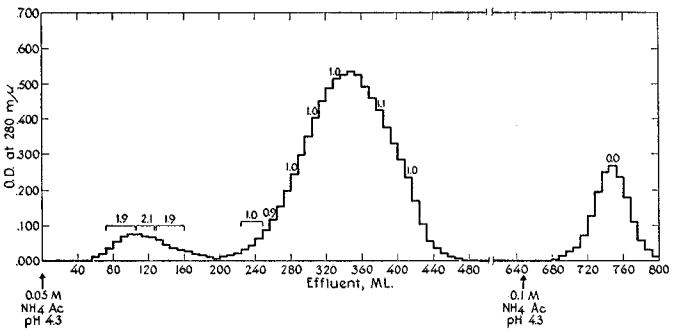

FIG. 1. Chromatographic separation of the HMC nucleotides of T2 phage DNA. The values shown refer either to the ratio of glucose to phosphate in single chromatographic fractions, or as in the case of the values over the brackets, represent the glucose to phosphate ratios in fractions which were pooled as indicated, then concentrated before analysis.

TABLE II

Distribution of hydroxymethylcytosine nucleotides in DNA from bacteriophages T2, T4, and T6

Phage*	Diglucosylated dHMP	Monoglucosylated dHMP	Nonglucosylated dHMP	Recovery of HMC of DNA as mononucleotide†
	% of total dHMP			%
T2	6	69	25	97
	6	70	24	96
	5	67	28	99
T4	<0.5	100	<0.5	93
T6	72	3	25	90
	72	4	24	88

* The data for T2 represent three separate analyses carried out on two different phage preparations. The data for T6 were obtained from two different phage preparations.

† Calculated assuming an HMC concentration of 0.17 mole per mole of DNA phosphate (5).

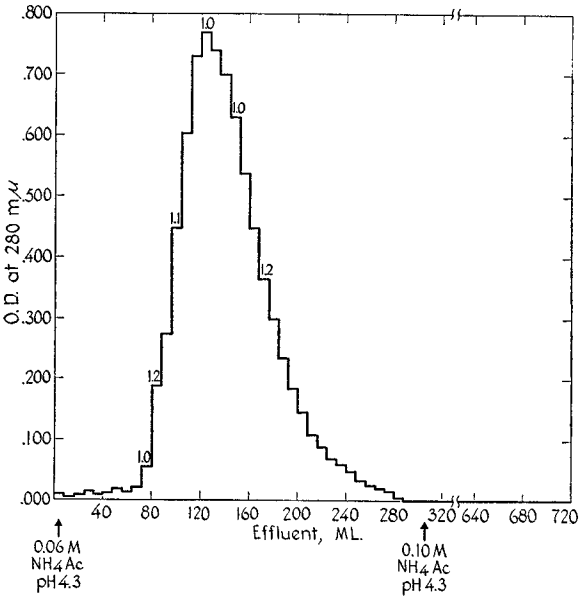

FIG. 2. Chromatogram showing the HMC nucleotide of T4 phage DNA.

RESULTS

In confirmation of the reports of several workers (1–4), the DNA's from coliphages T2, T4, and T6, although containing identical amounts of HMC, showed marked differences in their glucose contents (Table I). Thus, T2 DNA contains 0.82 mole of glucose per mole of HMC, T4 DNA contains approximately equimolar amounts of glucose and HMC, and T6 DNA contains 1.45 moles of glucose per mole of HMC.

HMC Nucleotides in T2 DNA—T2 DNA enzymatically degraded to mononucleotides as described under "Materials and Methods" was found to contain three distinct HMC nucleotides differing from each other only in their glucose contents (Fig. 1). The three peaks shown all had the spectral properties characteristic of an HMC nucleotide and contained, respectively, 2, 1, and 0 moles of glucose per mole of phosphate. As indicated in Table II the nucleotide bearing 1 mole of glucose was the predominant one; the value of 70% of the total HMC is in good agreement with the value reported by Sinsheimer on the basis of partial venom diesterase digests of T2 DNA (1). All three analyses of T2 DNA demonstrate the presence of a small but significant amount of the diglucosylated nucleotide (5 to 6% of the total). The remainder of the HMC (24 to 28% of the total) was present in the nonglucosylated form. In each of the analyses more than 95% of the HMC of the DNA which was enzymatically degraded was recovered in the form of the 5'-mononucleotide. Although the presence of other HMC nucleotides in these T2 phage preparations, for example polyglucosylated forms, cannot be entirely ruled out by these studies, the constancy of the glucose to phosphate ratios of individual fractions within a given chromatographic peak (Fig. 1), and the nearly complete recovery of HMC makes their existence unlikely.

HMC Nucleotides in T4 DNA—In contrast to T2, the chromatographic analysis of a T4 DNA digest revealed the presence of only the monoglucosylated HMC nucleotide (Fig. 2). Di- and nonglucosylated HMC, if present, would constitute less than 1% of the total HMC in T4 DNA (Table II). This finding is in agreement with the results of Volkin (2) and of Sinsheimer (18),

who observed only the monoglucosyl dHMP in partial enzymatic digests of DNA from T4 phage.

HMC Nucleotides in T6 DNA—As in the case of the T2, T6 DNA contains three distinct HMC nucleotides bearing, respectively, 2, 1, and 0 moles of glucose per mole of phosphate (Fig. 3). Again one-fourth of the total HMC is in the nonglucosylated form. In contrast to the T2 DNA, however, the HMC nucleotide containing 2 moles of glucose per mole of phosphate is present in highest concentration (72% of the total HMC); the monoglucosyl dHMP is present to the extent of only 3 to 4% of the total (Table II). Lichtenstein and Cohen (4), and Jesaitis (19) have previously reported that T6 DNA contains in addition to the diglucosylated HMC nucleotide, smaller amounts of the mono- and nonglucosylated nucleotides.

Linkage of Glucose to HMC in Monoglucosyl dHMP—It has been suggested by Sinsheimer (1) and by Volkin (2) that the glucose attached to the HMC of the DNA from phages T2 and T4 is bound in an *O*-glycosidic linkage to the 5-hydroxymethyl group of the pyrimidine. In an attempt to define more precisely the nature of these glycosidic linkages, their susceptibility to α- and β-glucosidases was examined.

The glucose of monoglucosyl dHMP from T2 DNA was found to be completely resistant to hydrolysis by the α-glucosidase from *S. italicus*. It was, however, quantitatively split from HMC by the α-glucosidase when the nucleotide was first dephosphorylated by the action of human semen monoesterase. The glucose bound to the HMC nucleotide or nucleoside was completely resistant to the action of β-glucosidase (Fig. 4).

The amount of nonglucosylated HMC nucleoside produced by the action of the α-glucosidase was found to be equivalent to the amount of free glucose formed. When 98% of the bound glucose had been released from the HMC nucleoside as shown in Fig. 4, the reaction was stopped by the addition of 2 volumes of cold 10% trichloroacetic acid. The precipitated protein was removed by centrifugation and the supernatant fluid was extracted 4 times with ether to remove the trichloroacetic acid. After the solution was reduced to about $\frac{1}{10}$ its original volume, one aliquot was taken for a glucose analysis by the hexokinase-Zwischenferment assay and a second aliquot was applied to Whatman No. 4 paper and chromatographed with the use of the ethanol-acetic acid solvent as described by Loeb and Cohen (20). After development of the paper chromatogram, only a single nucleoside appeared, that corresponding to the nonglucosylated derivative. The nucleoside was eluted from the paper with 0.1 N HCl and its concentration determined with the use of a molar extinction coefficient at 284 mμ of 13.2×10^3. The amount of HMC nucleoside recovered, 0.107 μmole, agreed with the free glucose (0.102 μmole) present in a comparable aliquot of the reaction mixture. These results indicate that the glucose in monoglucosyl dHMP from T2 DNA is bound to the hydroxymethyl group in an α-glycosidic linkage (Fig. 5).

T4 DNA, in contrast, contains two distinct monoglucosylated HMC nucleotides, one in which the glucose is bound in an α-glycosidic linkage and a second in which the linkage is in the β configuration. As shown in Fig. 6, incubation of the monoglucosylated nucleoside from T4 with the α-glucosidase resulted in the liberation of 67% of the total glucose; further addition of enzyme or longer incubation did not increase this value. However, addition of β-glucosidase to the reaction mixture resulted in the prompt liberation of the remainder of the glucose.

An experiment in which the monoglucosylated nucleoside from

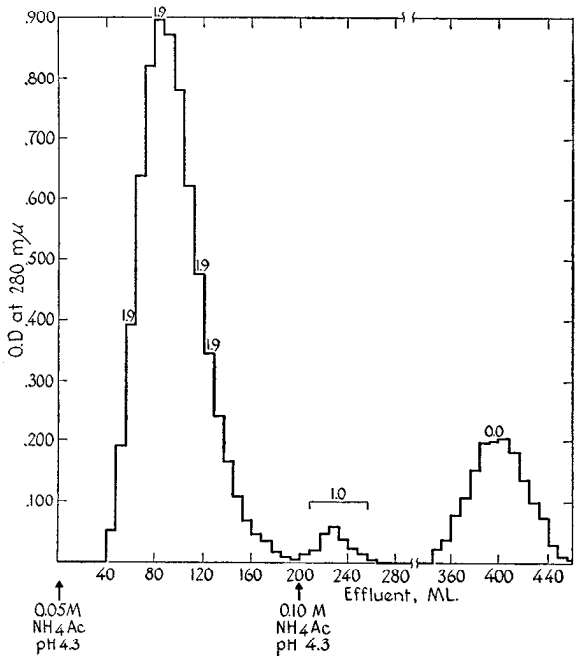

FIG. 3. Chromatographic separation of the HMC nucleotides of T6 phage DNA.

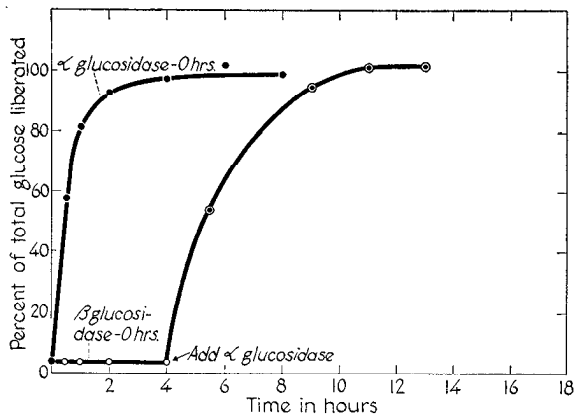

FIG. 4. Release of glucose from monoglucosyl HMC nucleoside from T2 DNA by α- and β-glucosidases. The reaction mixture for incubation with α-glucosidase contained 0.48 μmole of monoglucosyl HMC nucleoside, 20 μmoles of potassium phosphate buffer, pH 6.5, 0.4 μmole of cysteine, and 1000 units of α-glucosidase (DEAE fraction) in a volume of 1.55 ml. Incubation was at 37°; at the indicated times, aliquots (0.1 ml) were withdrawn, added to 0.4 ml of water, and heated for 5 minutes at 100°. The precipitated protein was removed by centrifugation, and 0.3 ml of the supernatant fluid was assayed for glucose by the hexokinase-Zwischenferment assay. The initial value of 4% free glucose is attributable to the presence of some α-glucosidase activity in the semen phosphomonoesterase used to prepare the nucleoside; this preparation contained no detectable β-glucosidase activity.

The reaction mixture for incubation with β-glucosidase contained 0.30 μmole of monoglucosyl HMC nucleoside, 20 μmoles of potassium phosphate buffer, pH 6.5, and 2200 units of β-glucosidase (ammonium sulfate fraction) in a volume of 1.3 ml. At the indicated times, aliquots (0.1 ml) were removed and assayed for free glucose as above. At 4 hours, 0.6 ml was withdrawn from the incubation mixture; 500 units of α-glucosidase (DEAE fraction), 0.25 μmole of cysteine, and 10 μmoles of potassium phosphate buffer, pH 6.5, were added and the volume adjusted to 1.0 ml. Aliquots (0.1 ml) were removed as indicated and assayed as before for free glucose.

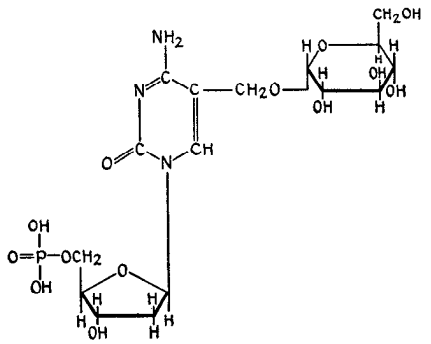

FIG. 5. Postulated structure of monoglucosyl dHMP from T2 DNA.

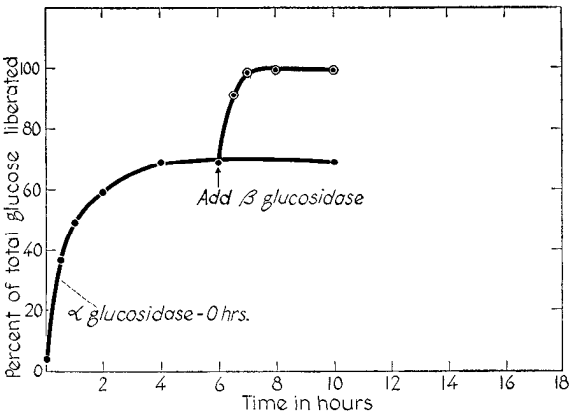

FIG. 6. Release of glucose from monoglucosyl HMC nucleoside from T4 DNA by α- and β-glucosidases. The incubation mixture for the reaction with α-glucosidase contained in a volume of 0.8 ml, 0.28 μmole of monoglucosyl HMC nucleoside, 10 μmoles of potassium phosphate buffer, pH 6.5, 0.2 μmole of cysteine, and 700 units of α-glucosidase (DEAE fraction). Incubation was at 37°; at the indicated times, aliquots (0.08 ml) were removed and analyzed for free glucose as described under Fig. 4.

At 6 hours, 0.3 ml of the reaction mixture was withdrawn and 1400 units of β-glucosidase (ammonium sulfate fraction) were added together with 5 μmoles of potassium phosphate buffer, pH 6.5. The volume was adjusted to 0.6 ml, and aliquots (0.1 ml) were again removed as indicated and assayed for free glucose.

T4 was treated first with the β-glucosidase and then with α-glucosidase resulted in the release of 30% of the total glucose by the first enzyme and the remaining 70% by the second.

In the case of the monoglucosylated HMC nucleoside from T6 DNA, determination of the glycosidic linkage was hindered by the small amounts of material available. One experiment, however, suggested that most (>80%) of the glucose was bound in the α configuration.

Linkage of Diglucosyl Unit in Diglucosyl dHMP—The diglucosyl unit of diglucosyl dHMP from T6 is a disaccharide in which the two glucose residues are linked to each other in a β linkage and to the hydroxymethyl group of HMC in an α linkage; the evidence for this is shown in Fig. 7. No detectable glucose[2] was liberated upon incubation of the diglucosyl-

[2] Neither free glucose nor a diglucosyl unit was released by the action of the α-glucosidase. Incubation of the diglucosylated HMC nucleoside first with the α-glucosidase, followed by heating of the reaction mixture to inactivate this enzyme, then incubation with β-glucosidase, resulted in the liberation of only 50% of the total glucose. Had a diglucosyl unit first been removed from the nucleoside by the α-glucosidase, 100% of the glucose would have been released upon addition of the β-glucosidase.

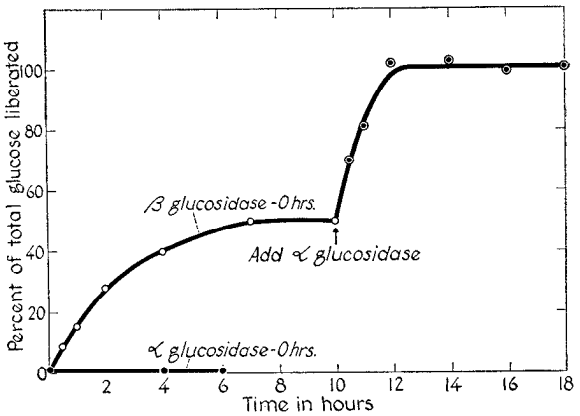

FIG. 7. Release of glucose from diglucosyl HMC nucleoside from T6 DNA by α- and β-glucosidases. The reaction mixture for incubation with β-glucosidase consisted of 0.33 μmole of diglucosyl HMC nucleoside, 20 μmoles of potassium phosphate buffer, pH 6.5; and 2000 units of β-glucosidase (ammonium sulfate fraction), in a final volume of 1.25 ml. Incubation was at 37°; at the indicated times, aliquots (0.1 ml) were removed and assayed for free glucose as described for Fig. 4.

At the end of 10 hours, 0.56 ml of the reaction mixture was withdrawn; 700 units of α-glucosidase (DEAE fraction), 8 μmoles of potassium phosphate buffer, pH 6.5, and 0.3 μmole of cysteine were added. The volume was adjusted to 1.2 ml and aliquots (0.1 ml) were again removed and assayed for free glucose.

The reaction mixture for incubation with α-glucosidase consisted of 0.20 μmole of diglucosyl HMC nucleoside, 20 μmoles of potassium phosphate buffer, pH 6.5, 0.3 μmole of cysteine, and 500 units of α-glucosidase (DEAE fraction) in a final volume of 1.0 ml. Aliquots (0.1 ml) were removed at zero time, 4 hours, and 6 hours and assayed for free glucose.

ated nucleoside with α-glucosidase; however, 50% of the total glucose was released by the action of the β-glucosidase. The remaining 50% could then be liberated by the action of the α-glucosidase.

The same result was obtained with the diglucosylated nucleoside from T2, suggesting that the disaccharides in the diglucosyl dHMP from T2 and T6 are the same. In neither case, however, was the position of the disaccharide linkage established.[3]

DISCUSSION

The availability of an enzyme which can quantitatively degrade the glucosylated DNA's from the T-even coliphages to their constituent mononucleotides has for the first time permitted an analysis of all their HMC nucleotides. This analysis has, in general, confirmed the conclusions drawn by Sinsheimer (1), Volkin, (2), Lichtenstein and Cohen (4), and Jesaitis (3) regarding the relative proportions of the various HMC nucleotides in these DNA's based on their studies of partial venom diesterase digests. The studies presented here demonstrate a number of additional features of interest concerning the nucleotide composition of these viral DNA's. First, T2 has been found to contain the diglucosylated HMC nucleotide as well as the mono- and nonglucosylated forms. Secondly, T4 DNA has been shown to contain two monoglucosylated HMC nucleotides, one in which the glucose is linked to the hydroxymethyl group of the pyrimi-

[3] There are four known β-linked disaccharides in which both monosaccharide units are glucose: cellobiose (1,4); gentiobiose (1,6); laminaribiose (1,3); and sophorose (1,2).

dine in the α configuration and a second in which the linkage is in the β configuration. This is in contrast to the situation in T2 in which there is only a single type of monoglucosylated HMC, that in which the glucose is bound in the α configuration. Thirdly, the diglucosyl unit of T6 and T2 DNA has been identified as a disaccharide in which the two glucose residues are linked to each other in a β linkage and to the hydroxymethyl group of the HMC in an α linkage. The position of the disaccharide linkage was not determined.

Genetic studies (19, 21, 22) have demonstrated that the proportion of glucose to phosphate in the T2, T4, and T6 DNA's is a characteristic, inheritable property of each phage type. Furthermore, the glucose content of the nucleic acid segregates from the host range marker in crosses between these phages. Cocito and Hershey (23) have recently reported that the glucose of phage T2 is transferred from parental to offspring phage with the same efficiency as other DNA constituents. It is therefore reasonable to suppose that the presence of a glucosyl or diglucosyl unit linked to a particular HMC residue may in some way contribute to the genetic information embodied in the nucleotide sequence of which the HMC residue is a part. Streisinger *et al.* (21, 22) have reported the isolation of strains from crosses of phages T2 and T4 which showed glucose contents intermediate between T2 and T4. Jesaitis (19) has made similar observations with strains derived from crosses of T2 and T6. It would be of interest to examine enzymatic digests of the DNA of these hybrid phages to determine the way in which their altered glucose contents are reflected in the distribution of the various glucosylated and nonglucosylated HMC nucleotides.

Kornberg *et al.* (24) have reported the enzymatic glucosylation of HMC-containing DNA's by direct transfer of glucose from uridine diphosphate glucose to the HMC of the DNA in a reaction catalyzed by enzymes detectable only in extracts of phage-infected bacteria. Of further interest is the observation[4] that extracts of T4-infected *E. coli* contain two distinct and physically separable enzymes, both of which monoglucosylate DNA containing HMC. Recent experiments[4] suggest that one of these enzymes adds glucose to the HMC of the DNA in a β linkage and the second, which has its counterpart in an enzyme present in T2-infected extracts, adds the glucose in the α configuration. The presence in T4 DNA of the α- and β-linked glucose residues is therefore consistent with the induction of two specific glucosylating enzymes upon infection of *E. coli* with T4 bacteriophage.

SUMMARY

The nucleic acids from bacteriophages T2, T4, and T6 have been quantitatively degraded to their 5′-mononucleotide components with a phosphodiesterase from *Escherichia coli*. The non-, mono-, and diglucosylated hydroxymethylcytosine (HMC) nucleotides were separated, their relative proportions determined, and the glucose linkages identified. For T2 it was found that, of the total HMC content, 25% is nonglucosylated, 70% is monoglucosylated, and 5% is diglucosylated. In both the monoglucosylated and diglucosylated HMC, glucose is bound to the hydroxymethyl group in an α-glycosidic linkage. In the diglucosylated form, the second glucose is bound to the first by a β linkage. In T4 all the HMC is in the monoglucosylated form, but 70% has the glucose attached in the α-configuration and 30% has a β linkage. In T6 25% of the HMC is nonglucosylated, 72% is diglucosylated, and 3% is monoglucosylated, with the linkages the same as in T2.

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[4] Personal communication from S. R. Kornberg.

REFERENCES

1. Sinsheimer, R. L., *Science*, **120**, 551 (1954).
2. Volkin, E., *J. Am. Chem. Soc.*, **76**, 5892 (1954).
3. Jesaitis, M. A., *J. Exptl. Med.*, **106**, 233 (1957).
4. Lichtenstein, J., and Cohen, S. S., *J. Biol. Chem.*, **235**, 1134 (1960).
5. Wyatt, G. R., and Cohen, S. S., *Biochem. J.*, **55**, 774 (1953).
6. Cohen, S. S., *Cold Spring Harbor symposia on quantitative biology, Vol. 18*, Long Island Biological Association, Cold Spring Harbor, Long Island, New York, 1953, p. 221.
7. Lehman, I. R., *J. Biol. Chem.*, **235**, 1479 (1960).
8. Halvorson, H., and Ellias, L., *Biochim. et Biophys. Acta*, **30**, 28 (1958).
9. Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 751 (1956).
10. Adams, M. H., *Bacteriophages*, Interscience Publishers, Inc., New York, 1959.
11. Herriott, R. M., and Barlow, J. L., *J. Gen. Physiol.*, **36**, 17 (1952–53).
12. Anderson, E. H., *Proc. Natl. Acad. Sci. U. S.*, **32**, 120 (1946).
13. Sinsheimer, R. L., and Koerner, J. F., *Science*, **114**, 42 (1951).
14. Park, J. T., and Johnson, M. J., *J. Biol. Chem.*, **181**, 149 (1949).
15. Kornberg, A., *J. Biol. Chem.*, **182**, 805 (1950).
16. Morris, D. L., *Science*, **107**, 254 (1948).
17. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
18. Sinsheimer, R. L., *Proc. Natl. Acad. Sci. U. S.*, **42**, 502 (1956).
19. Jesaitis, M. A., *Federation Proc.*, **17**, 250 (1958).
20. Loeb, M. R., and Cohen, S. S., *J. Biol. Chem.*, **234**, 364 (1959).
21. Streisinger, G., and Weigle, J., *Proc. Natl. Acad. Sci. U. S.*, **42**, 504 (1956).
22. Streisinger, G., Mukai, F., Kim, S. W., *Carnegie Institution of Washington Year Book, Vol. 58*, Carnegie Institution, Washington, D. C., 1959, p. 432.
23. Cocito, C., and Hershey, A. D., *Biochim. et Biophys. Acta*, **37**, 543 (1960).
24. Kornberg, A., Zimmerman, S. B., Kornberg, S. R., and Josse, J., *Proc. Natl. Acad. Sci., U. S.*, **45**, 772 (1959).