The compositions of the nucleic acids of the T even bacteriophages, T2, T4, and T6 have been shown to be identical with respect to their purine and pyrimidine bases within the accuracy of existing analytical methods for these bases (1). However, these viruses contain the unusual pyrimidine, 5-hydroxymethyl cytosine, which provides a unique opportunity for chemical variability in the composition of these nucleic acids. Cohen found (2) that enzymatic digestion of phage nucleic acids by pancreatic deoxyribonuclease and intestinal phosphatase released large amounts of the deoxyribonucleosides of adenine, guanine, and thymine but only a very small proportion of the HMC deoxyribonucleoside, although a similar digestion of thymus deoxyribonucleic acid leads to a quantitative degradation to the deoxyribonucleosides. It was suggested that the structural relations of HMC in some way conferred resistance to the phosphatase action of these enzymes. The HMC deoxyribonucleoside was released by phosphatase after a preliminary acid hydrolysis (2).

It was reported in 1940 that purified preparations of T2r+ bacteriophage contained hexose which reacted like glucose (3). Jesaitis and Goebel (4) have reported on the presence of hexose in T4 preparations and Jesaitis (5) first demonstrated the association of glucose with the viral nucleic acid.

In 1954 Volkin (6) and Sinsheimer (7) showed independently that the DNA's of T4r and T2r+ bacteriophages, respectively, could be hydrolyzed, albeit incompletely, by pancreatic DNase and by venom phosphodiesterase to liberate small amounts of a nucleoside which contained both HMC and glucose. The glucose was present as an O-glycoside of the 5-hydroxymethyl substituent on the pyrimidine. In the digests of T4r+ nucleic acid the ratio of glucose to HMC in various fractions was 1:1 (6). However, two HMC nucleotides were isolated by ion exchange chromatography from digests of T2r+ DNA, one of which contained a mole of glucose per mole of HMC whereas the other was free of the hexose (7). It was reported that the monoglucosyl HMC nucleotide is dephosphorylated by a 5'-nucleotidase at a markedly slower rate than is the monoglucosylated dHMP (7).

Jesaitis (8) has analyzed the DNA of wild type r strains of T2, T4, and T6 and has shown that the three strains, which contain essentially similar amounts of HMC, contain different amounts of glucose in the amount of approximately 0.8, 1.0, and 1.6 moles of glucose per mole of HMC, respectively. He has isolated small amounts of a diglucosyl derivative of dHMP and of nonglucosylated dHMP from enzymatic digests of the DNA of T6r+ (9). The isolation of the diglucosyl derivative of HMC deoxyribonucleoside has recently been described by Loeb and Cohen (10).

Biosynthetic routes to the formation of some of these unique viral compounds have been demonstrated. Flaks and Cohen (11) have described the biosynthesis of dHMP from dCMP by an enzyme produced only in Escherichia coli infected by T-even bacteriophages. The conversion of dHMP to the triphosphate and its enzymatic incorporation into DNA have been described by several laboratories (12-14). The monoglucosylated HMC nucleoside triphosphate does not seem to be active in this system (15). On the other hand Kornberg et al. (12) have described a monoglucosylation of DNA containing HMC by UDP-glucose in the presence of an enzyme derived from extracts of T2-infected E. coli. The glucose of T6r+ DNA is the sole constituent of virus DNA which is formed completely de novo after infection (16). This is in keeping with the requirement for UDP-glucose in formation of virus DNA, whereas other units, purines, pyrimidines, etc. are in part derived from preformed host constituents.

The exact role of glucosyl moieties in determining the specificity of the phage nucleic acids in heredity is not known. A relation has been observed between the extent of glucosylation of HMC nucleotides in phage DNA and the efficiency with which phage infection leads to virus multiplication (16). This can conceivably be related to survival of phage polynucleotide sequences within the host, as postulated earlier (2, 17). This laboratory has long been interested in the nature of the r mutation affecting cell lysis and we have examined various properties of pairs of r+ and r mutants. No differences had been observed between the base compositions of the parent and mutant strains. However, preparations of r strains and of the DNA derived from these strains were obtained which contained larger amounts of glucose (18) than did similar preparations of r+ phages and their DNAs, whose glucose contents were comparable to the values reported by other workers (6-8). Variations in the glucose content of the r preparations and their isolated DNA's suggested the possibility that the extra glucose might be derived from degradation products of bacteria lysed more extensively by r phages (18) and it was felt that to test this point it would be necessary to seek and possibly exclude the presence of HMC nucleotides containing polyglucosyl units.

Existing enzymatic methods of releasing HMC nucleotides gave low yields of such compounds and although considerable
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effort was expended in devising chemical techniques to increase the yield of HMC mononucleotides; no such method was indeed devised. Accordingly in lieu of a better approach the DNA of pairs of r+ and r strains were degraded extensively by pancreatic DNase and venom phosphodiesterase and the incompletely hydrolyzed digests were analyzed by ion exchange chromatography. These analyses are presented in this paper, as are a number of properties of various HMC nucleotides.

EXPERIMENTAL

Materials and Methods

Biological Systems—The preparation of cultures of E. coli strain B in various media and their use in the production of various T-even bacteriophages have been described (19, 20), as have been the isolation and properties of r+ and r strains of the T2, T4, and T6 bacteriophages used in these studies. Dr. Seymour Benzer of Purdue University has analyzed our r strains and has informed us that of the strains used in the earlier studies (19, 20) T2r was of the rI group, whereas T4r and T6r are of the rI group. Starting with our T2r+ stock (T2C) Dr. Benzer kindly isolated a T2r+1 (designated T2C8 in his files) and this strain was used in this analytical work.

Chemical Methods; Preparation and Degradation of DNA—Preparations of phage containing 100 to 200 µmoles of DNA phosphorus were degraded by urea (10) and after extensive deproteinization, fibrous preparations of sodium salts of DNA were precipitated by ethanol and dried in a vacuum over P2O5. The solids were soaked overnight at 4° in H2O (about to 2 mg per ml) and the very viscous solution was subsequently worked slowly through the digest. The pH was maintained at pH 6.5 with dilute NaOH.

Crystalline pancreatic DNase (Worthington) (1 mg) was added per 100 mg of DNA preparation. The pH, determined electrometrically with a glass electrode and pH meter, was followed continuously at 37° with CO2-free Na2 bubbling slowly through the digest. The pH was maintained at pH 6.5 by frequent additions of dilute standard alkali and the digestion was continued until no pH change occurred for 30 minutes. In each digestion another milligram of DNase was added, and the reaction was continued for another hour, although no additional pH change was ever observed. The digests were then adjusted to pH 9.2 with NaOH and purified phosphodiesterase2 was added. This enzyme was isolated (21) from 100 to 200 mg quantities of the venom of Crotalus adaimanteus, obtained from the Ross Allen Institute, Silver Springs, Florida. The purified phosphodiesterase was demonstrated to be essentially free of phosphomonoesterase, as tested on DNase digests of thymus DNA. Phosphodiesterase in an amount equal to that derived from 40 mg of venom was added to an amount of digest equivalent to 100 mg of DNA, and the pH was maintained at pH 9.2 by addition of standardized alkali during digestion. Hydrolysis seemed to be complete in about 90 minutes. After another 30 minutes, added portions of diesterase did not increase hydrolysis. Thus the limitation in enzymatic cleavage did not seem to arise from the development of products inhibitory to the enzyme. However it was noted in one experiment with T2r+ DNA that repetitive treatment with DNase and phosphodiesterase did lead to a significantly higher yield of mononucleotides and particularly of the HMC nucleotides.

Analytical Methods—Analyses were made on the DNA solutions after digestion by DNase had reduced the viscosity of the solutions sufficiently to permit accurate pipetting. Phosphorus was determined by the method of King (22) and deoxyribose by the diphenylamine reaction (23). The estimation of glucose by the anthrone method has been described (16). Since deoxyribose is reactive in this reaction, it is necessary to attempt suitable corrections for the color contributed by nucleotides containing this substance. The estimation of this correction is difficult since not only does purine-bound deoxyribose react, as in the diphenylamine reaction, but pyrimidine-bound deoxyribose does also to a slight extent. The extent of the correction may be made relatively accurately with mono- or dinucleotide fragments whose base compositions are known and whose monoglycosylated components may be assembled in a test mixture. In most estimations of the glucose content of the mononucleotides, dCMP was used as a blank. However the correction is necessarily less accurate with digests or larger polynucleotide fragments. With the latter types of material it is unlikely that the determination of glucose content by this colorimetric method is more accurate than ±10 to 15%.

Ion Exchange Chromatography: The enzymatic digests were fractionated by the method of Sinsheimer and Koerner (24). Digests containing 100 to 200 µmoles of DNA phosphorus, adjusted to pH 10, were applied to Dowex 1-aceate columns (10 cm × 1 cm, 4% cross-linked) at concentrations of 0.01 to 0.02 M with respect to total anion. The preparation of the column has been described (23). The column filtrate or eluate was collected at a flow rate of 0.2 to 0.4 ml per minute in 5 ml fractions on a Gilson fraction collector, equipped with an ultraviolet absorption spectrophotometer, continuously recording percentage of transmission at 2537 A. The initial filtrate was washed through with 0.01 M NH4OH and the column was then washed with H2O. Elution was carried out with ammonium acetate buffers adjusted to pH 4.3. These were used successively at the following concentrations: 0.01 M, 0.03 M, 0.06 M, 0.1 M, 0.2 M, 0.3 M, 0.5 M, 1 M, and 2 M. With each eluent, a minimum of 100 ml of buffer was applied; and if a component was eluted, the eluent was not changed until the concentration of component in the eluate had fallen to essentially zero. After completing the elution of a component at the end of a day, the flow of eluant was frequently arrested until the following morning. On beginning the flow of buffer on the next morning a small amount of guanine usually came off quite rapidly, reflecting the small but significant liability of the deoxyribosyl linkage to guanine at pH 4.3 at room temperature.

Fractions containing the separated mononucleotides were pooled after a more extensive spectrophotometric examination and were concentrated by distillation under reduced pressure. Fractions lacking appreciable ultraviolet absorption were also pooled and concentrated. Phosphorus analyses were made of all fractions; phosphorus was present only in fractions characteristic of the known mononucleotides and larger components of the digest. After estimating the yields of the mononucleotides, dTMP, dAMP and dGMP, which were shown to have dryness in a vacuum and finally lyophilized to minimize the ammonium acetate content of the preparations.

The yields of the mononucleotides of HMC were estimated

2 We are indebted to Dr. J. F. Koerner for telling us of an improved isolation method for this enzyme before its publication.
Enzymatic Degradation of phage DNA

<table>
<thead>
<tr>
<th>Sample of DNA</th>
<th>Effect of DNase</th>
<th>Phosphodiesterase after DNase</th>
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<tr>
<td></td>
<td>Mole H⁺ liberated per mole DNA-P</td>
<td>Increment in ultraviolet absorption*</td>
</tr>
<tr>
<td>T₂r⁺</td>
<td>0.08</td>
<td>34</td>
</tr>
<tr>
<td>T₂r⁻</td>
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<tr>
<td>T₄r⁻</td>
<td>0.12</td>
<td>36</td>
</tr>
<tr>
<td>T₆r⁺</td>
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<td>35</td>
</tr>
<tr>
<td>T₁₀r⁻</td>
<td>0.11</td>
<td>36</td>
</tr>
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<td>Thymus</td>
<td>54-38</td>
<td>15.4</td>
</tr>
<tr>
<td>E. coli</td>
<td>54</td>
<td>29.8</td>
</tr>
</tbody>
</table>

* These estimations were kindly made by Miss H. D. Barner.

Results

Extent of Enzymatic Digestion—The electrometric back titration of the digest to maintain digestion at a constant pH provided a measure of the extent of cleavage of the DNA chains. It can be seen in Table I that the extent of hydrolysis of phage DNA by DNase was of the order of 10% of the phosphodiester bonds, or about half that observed with DNA derived from other materials (26).

The subsequent degradation by phosphodiesterase cleaved an additional 50 to 60% of the phosphodiester bonds as recorded in Table I. This amounts to a total of 60 to 70% of the total phosphodiester bonds, as contrasted to an essentially quantitative cleavage of thymus DNA (27) or a greater than 93% cleavage of T7 DNA (28) to mononucleotides by the same treatment. Thus the stepwise digestion of all the T-even phage nucleic acids by pancreatic DNase and Crotonus venom phosphodiesterase left 30 to 40% of the nucleotides in polynucleotide fragments. In contrast to the results with phage DNA an identical digestion of E. coli DNA and fractionation by ion exchange separation resulted in a 94% recovery in the form of mononucleotides as estimated on a phosphorus basis or a 98% recovery estimated spectrophotometrically.

Analysis of the ultraviolet absorption at 2800 A at 37° during digestion of DNA at about 30 µg per ml by DNase at 0.45 µg of DNase per ml in 0.05 M Tris buffer pH 7.5 and 0.02 M MgCl₂ revealed that the increase in this function for phage DNA was comparable to that observed with samples of thymus DNA and E. coli DNA, as presented in Table I. At lower concentrations of the enzyme, which revealed the markedly slower rate of degradation of the various phage DNA samples, as contrasted with thymus DNA, the increase in ultraviolet absorption finally attained was frequently less than 30% and appeared to reflect the inhibition of the enzyme by DNA and its degradation products. In any case, degradation of DNA by DNase carried to completion appeared to eliminate essentially all structural integrity responsible for the restriction of ultraviolet absorption in phage DNA.

Studies were also made of the sedimentation behavior of phage DNA and of DNase digests to determine whether the products of digestion of the phage DNA were markedly smaller than those of thymus DNA. Sedimentation velocity studies of solutions of phage DNA and digests (3 to 4 µg of DNA phosphorus per ml) were carried out in a Spinco model E ultracentrifuge equipped with ultraviolet absorption optics (29). A Spinco Analytrol was used to read the films. The analysis of these densitometer readings to estimate sedimentation constants has been described (29).

The s rates were corrected to that for water at 20°. Samples of intact DNA and phage digests having an optical density at 260 mµ of 0.7 to 0.9 were analyzed in 0.1 M Tris buffer at pH 7.6. The distribution of s rates of these samples was determined. The DNA's were observed to be somewhat less heterogeneous and to possess somewhat higher s rates than are obtained for most samples of thymus DNA. These samples also possessed a considerably higher ssp 0, extrapolated to infinite dilution and zero shear gradient, than had thymus DNA. The s rate recorded in Table I is that of material at the midpoint of the sedimenting boundary. As recorded earlier (30) a bimodal distribution of ssp 0 rates was not observed for phage DNA. In the absence of data on the concentration dependence of the s rate and distribution function for phage DNA, and in the absence of data on the effect of the isolation procedure on the physical parameters of this material, the s rates for the DNA before digestion which are presented in Table I will not be discussed further.

In order to analyze the sedimentation of the slowly sedimenting DNase digests, use was made of the synthetic boundary cell (29). As can be seen in Table I, the ssp 50% rates of the DNase digests of phage DNA and other DNA are not significantly different, and indeed the spread of the boundaries is also quite similar. After a digestion with DNase carried to completion the s rates at the 50% point of the various boundaries were 1.1 to 1.4, and at the 90% point were 6 to 7. Thus digestion of phage DNA by pancreatic DNase did not leave a high molecular fraction comprising a significant portion of the initial DNA.

Analysis of Digests—The initial alkaline filtrate through the column usually contained a very small amount of deoxyribonucleotides and free bases. The ultraviolet absorption of this fraction, usually having a maximum at 255 to 260 mµ at pH 10 amounted to 0.5 to 1.5% of the total. This fraction, the H₂O washes and the eluate made with 0.01 M acetate pH 4.3 did not contain phosphorus.

The first nucleotides to be eluted contained HMC and three such nucleotides were observed. These contained in the order HMC, HMD, HDP, and HDM as recorded earlier (30) a bimodal distribution of ssp 0 rates was not observed for phage DNA. In the absence of data on the concentration dependence of the s rate and distribution function for phage DNA, and in the absence of data on the effect of the isolation procedure on the physical parameters of this material, the s rates for the DNA before digestion which are presented in Table I will not be discussed further.
of their appearance from digests of T6 DNA a diglucosyl moiety, a monoglucosyl moiety, and a final nucleotide lacking glucose. From such a digest (150 μmoles of P applied to the column) the diglucosyl dHMP could be eluted with 100 ml 0.03 M acetate (pH 4.3) and the monoglucosyl and nonglucosylated dHMP were eluted with 0.06 M buffer. Occasionally a column was obtained with which the elution of the diglucosyl nucleotide with 0.03 M acetate proceeded too slowly, and in this case 0.06 M acetate was used to elute the diglucosyl- and monoglucosyl-dHMP while 0.1 M acetate was used to elute dHMP. On this column dCMP is eluted with 0.1 M acetate; no trace of this compound was ever observed in these digesta. Records of the elution patterns of the dHMP nucleotides for digests of DNA of T6r+ and T6r are presented in Figure 1A and B, respectively. It can be seen that each digest contains three comparable components; these are the dHMP nucleotides noted above, and the properties of these materials will be considered in detail below.

As shown in Figure 1 and Table II, the monoglucosylated nucleotide is present only as a small fraction of the total dHMP nucleotide of which approximately 40% has been recovered in both cases. Jesaitis first discovered the existence of diglucosylated dHMP and nonglucosylated dHMP in T6r+ digests (9) but in these studies did not detect the presence of the small amount of the monoglucosylated nucleotide. He has since observed the three substances in T6 digesta in essentially the proportions described above (31).

The similarity of the patterns in studies of digests of several preparations of T6r+ DNA and in the T6r+ and T6r analyses suggest that the monoglucosyl dHMP is not an artifact arising from the degradation of the diglucosyl derivative. The quantitative variation between the two preparations with respect to their content of the monoglucosyl component, as reported in Table II, may arise in several ways. The analysis of a great many preparations of T6r+ DNA and in the T6r+ and T6r analyses suggested that the monoglucosyl dHMP nucleotides noted above, and the properties of these materials will be considered in detail below.

In Fig. 2A and B are presented the elution patterns of the dHMP nucleotides from digests of the DNA of T2r+ and T4r+, respectively. As can be seen in Fig. 2A, no trace of the diglucosyl derivative was obtained from T2r+ and only the monoglucosyl and nonglucosylated nucleotides were observed in similar amounts during elution with 0.06 M buffer. In the analyses reported in this figure, in which the high yield of 60% of the total

* For the purposes of estimating yields of recovery of dHMP nucleotides the total HMC content of T even phage DNA is assumed to be 0.16 mole HMC per mole phosphorus (1).
Nucleotides from Nucleic Acids of T2, T4, and T6 Bacteriophages

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As reported in Table II for both T4RF and T4R, only 20% of the total HMC was obtained in this form in each case. The separation of dTMP and dAMP was never entirely complete in this system in a single run. The elution was effected initially at 0.2 M acetate and a point was usually obtained where-in the elution of dTMP was almost complete but had not fallen to zero before dAMP began to be eluted. The degree of overlap did not involve more than 5% of either of the two nucleotides estimated spectrophotometrically. At the point where dTMP fell and dAMP appeared, the molarity of the buffer was increased to 0.3 M. The final mononucleotide, dGMP, was eluted at 0.5 M acetate buffer. Polynucleotide fragments were then eluted at 1.0 M, 1.5 M, and 2.0 M acetate. The columns were finally washed with 2 M HCl; this final eluate rarely contained significant amounts of phosphorus.

No other mononucleotides were detected by this analytical procedure. Digests of T6 DNA contained a dinucleotide of adenine and diglucosylated HMC which was eluted just before dGMP, as can be seen in Fig. 3. However it was not separated cleanly from dGMP in a single elution sequence. The properties of this fragment, comprising 7.5% of the total HMC, will be described below.

Properties of HMC Mononucleotides—The separated mononucleotides were lyophilized to minimize the ammonium acetate in the preparations and were stored at -20°. Further purification was effected by adsorption on and elution from charcoal as described by Pontis et al. (32). Yields were of the order of 50 to 70% of the nucleotide applied.

The dHMP nucleotides were characterized and differentiated by their composition, their ultraviolet absorption properties over a wide range of pH, chromatographic properties in two solvent systems, electrophoretic mobilities, and sensitivity to alkaline phosphatase.

The absorption maxima of the three dHMP nucleotides were found to be at 283 to 284 mp in 0.1 N HCl and at 275 mp at neutrality and in 0.1 N NaOH. The minima were at 245 mp in acid and at 255 mp at neutrality and in alkali. These properties and spectral changes in acid, neutral pH, and alkali affirmed the identity of ionizable groups in a common base in the three nucleotides. The pK of the amino groups of each nucleotide was estimated by the spectrophotometric method (33); in earlier studies by this method (10) the pK values of the HMC deoxyriboside and of the diglucosyl derivative of this deoxyriboside were found to be 3.5 to 3.6 and 3.4 to 3.5, respectively. In Figs. 4A, B, and C are presented the effects of pH on the various extinction ratios of the mononucleotides. In these figures the diglucosylated and non-
glucosylated nucleotides were derived from the T6rI preparation
while the monoglucosylated derivatives were obtained from the
T4rf preparation. The diglucosyl derivative was found to con-
tain 1.83 moles of glucose per mole of P, and the T4 derivative
had 1.02 moles of glucose per mole of P. Identical pK values
were obtained for the diglucosyl derivative derived from T6r+
(1.96 moles of glucose per mole of P) and from T6rI. It can be
determined from the figures that the pK values of the amino
group were 3.75 to 3.80 for the diglucosyl-dHMP, 3.6 to 3.75 for
the monoglucosyl dHMP, and 4.0 to 4.2 for free dHMP. In free
dHMP it may be inferred that the presence of the phosphate and
its propinquity to the amino group inhibit the loss of the proton
from \(-\text{NH}_2^+\), accounting for the higher pK of this group in
dHMP as contrasted to the deoxyribonucleoside. Substitution
at the \(-\text{CH}_2\text{OH}\) interposes a group between the phosphate and
\(-\text{NH}_2^+\) and reduces the pK of the latter. Thus the spectral
properties of the glucosylated compounds were quite diierent
from those of the nonglucosylated dHMP at the pH of elution
(pH 4.3) from the ion exchange column as a function of the dif-
fences in extent of dissociation of the amino groups of these
compounds.

The separated nucleotides were studied by descending paper
chromatography in two solvent systems, the isobutyrate-NH$_4$OH
system at pH 3.9 (34) and the ethanol-acetate-tetraborate sys-
tem (35). The electrophoretic motilities of the compounds were
also compared on paper in 0.2 M borate buffer at pH 9.2 in the
E. C. 105 electrophoresis apparatus at 22.2 volts per cm for 2
hours. The behavior in these systems readily distinguished the
compounds, as can be seen in Table III.

In the unfractionated digests of thymus DNA, chromatogra-
phy in the isobutyrate system easily reveals the four nucleotides
in the order of increasing \(R_f\): dGMP, dTMP, dCMP, and dAMP.
Similar analyses of digests of phage DNA clearly demonstrates
the deficiency of dCMP, although a faint spot attributable to
dHMP may be perceived in that area. In addition the gluco-
sylated dHMP derivatives are easily detected in the area between
polynucleotide fragments and the more mobile dGMP.

The sensitivities of the three HMC nucleotides and of dCMP
to hydrolysis by calf intestinal alkaline phosphatase (35) were
compared. Enzyme purified up to, but not including the acetone
precipitation step (9), was used. Four units of phosphatase in
0.025 ml were added to 0.33 \(\mu\) mole of nucleotide in 1.1 ml of 0.05
Chromatographic and electrophoretic properties of HMC nucleotides

<table>
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<tr>
<th>Compound</th>
<th>Paper chromatography</th>
<th>Paper electrophoresis</th>
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<tr>
<td></td>
<td>Isobutyrate</td>
<td>Ethanol-tetraborate</td>
</tr>
<tr>
<td>dCMP</td>
<td>0.30-0.45</td>
<td>0.18</td>
</tr>
<tr>
<td>Diglucosyl</td>
<td>0.15-0.18</td>
<td>0.41-0.47</td>
</tr>
<tr>
<td>dHMP</td>
<td>0.27-0.28</td>
<td>0.63-0.64</td>
</tr>
<tr>
<td>Monoglucosyl</td>
<td>0.35-0.40</td>
<td>0.88-0.90</td>
</tr>
<tr>
<td>dHMP</td>
<td>0.27-0.30</td>
<td>0.69-0.76</td>
</tr>
</tbody>
</table>

Fig. 5. A comparison of the enzymatic dephosphorylation by alkaline phosphatase of dCMP and various glucosylated derivatives of dHMP.

Tris buffer pH 9.2 containing 0.03 M Mg++ and the mixture was incubated at 37°. Aliquots were removed, acidified, and inorganic P was determined. In Fig. 5, it can be seen that dHMP is dephosphorylated at a markedly slower rate than is dCMP. Glucosylation sharply reduces the rate of dephosphorylation of dHMP to 10% or less of the rate at which dCMP is cleaved. It is concluded that the fraction is a dinucleotide consisting of dAMP and the diglucosylated dHMP.

Treatment with alkaline phosphatase converted slightly more than 50% of the total phosphorus to inorganic P. Hydrolysis at 100° with N HCl for 1 hour also cleaved half of the total P.

Note added in proof: Four structural possibilities exist for the dinucleotide. These are d-pApH, d-pHpA, d-ApHp, and d-HpAp, where H represents the diglucosyl hydroxymethyldeoxyribose. The last two structures are excluded by the method of preparation. The second possibility would be expected on acid hydrolysis or treatment with Burton's reagent to lead to the formation of the diphasate of the pyrimidine nucleoside [Burton, K. and Petersen, G. B., Biochim. et Biophys. Acta, 26, 667 (1957) and Potter, J. L. and Laskowski, J. L., J. Biol. Chem., 224, 1263 (1959)], whereas the first should lead to the formation of the 5'-monophosphate of the pyrimidine nucleoside. After treatment with Burton's reagent, a compound was found whose Rp in isobutyrate NHCl and electrophoretic mobility in borate were those of the diglucosyl dHMP. It is concluded that the nucleotide probably has the structure d-pApH.

DISCUSSION

No evidence could be obtained in these analyses to show that the DNA of a mutant r phage differed significantly in its content of glucosylated dHMP nucleotides from the DNA of the parent r+ phage. However these experiments revealed only a part of the total HMC of the DNA as mononucleotides and it might be imagined that the HMC attached to polynucleotide fragments contained other types of glucosylated nucleotides which were even more resistant to enzymatic degradation. It is of interest in this connection that Lehman has recently described (38) a nuclease derived from E. coli which was capable of complete conversion of a partially digested phage DNA to mononucleotides and in a hydrolysate of T2 r+ DNA was able to detect a small amount of the diglucosylated dHMP. From the yield of non-glucosylated dHMP from T2 r+ DNA, in Analysis B, recorded in Table II, it may be calculated that the glucosyl content of T2 DNA is too high to be accounted for by monoglucosylated dHMP alone and leads one to postulate other polyglucosyl derivatives. The application of the new nuclease to r+ and r DNA should be most helpful in settling the question originally posed.

Although the addition of glucosyl moieties to HMC deoxyribo-nucleoside did not affect the pK of the amino group to a marked extent, it is clear that the nucleotides present a different picture. The pK of the amino group seems to be similar in dHMP and in dCMP, the former being 4.0 to 4.2 and the latter at 4.2. The addition of one or more glucosyl moieties to dHMP reduces the pK very significantly to 3.6 to 3.8, accounting in part for the ease of separation of the glucosyl derivatives from the nonglucosylated nucleotide. However the diglucosyl and monoglucosyl derivatives are also cleanly separated in elution from Dowex 1-acetate despite the similarities of their pK values. This phenomenon of the effect of glucose in decreasing affinity for the resin may also be observed in the relatively early elution of the diglucosylated dinucleotide in T6 digests, when other dinucleotides from other

purine and pyrimidine nucleotides. Hydrolysis for 1 hour at 60° in N HCl, followed by chromatography in butanol-NHClOH (37), revealed adenine as the sole mobile base. The glucose content of the material was estimated with a mixture of equimolar amounts of dAMP and dCMP as the blank. The substance contained 1.1 moles of glucose per mole of P. It was concluded that the fraction is a dinucleotide consisting of dAMP and the diglucosylated dHMP.

Properties of Dinucleotide—The fraction present in T6 DNA digests which was eluted by 0.5 M acetate just before the elution of dGMP was concentrated and lyophilized. A solution of this material was subjected to various analyses. It did not chromatograph as a compact spot in the isobutyrate system. Spectra were measured at pH 1, 7, and 13 and absorption maxima were observed at 270 mp, 266 mp, and 265 mp, respectively. The absorbance ratios were those of a 1:1 mixture of dAMP and dHMP. The material contained 0.5 mole of reactive deoxyribose per mole of P, suggesting the presence of equal amounts of
digests are not detectable before release of all of the mononucleotides. The effects of such a substituent are also noteworthy in paper chromatography in the isobutyrate system in which affinity for the aqueous phase is increased by addition of glucose. In paper electrophoresis in borate at pH 9.2 the increase in molecular volume of the nucleotides resulting from addition of glucose decreases electrophoretic mobility and more than compensates for the effect of borate which increased net charge.

**SUMMARY**

The deoxyribonucleic acids of three pairs of parent and mutant phages, T2r+ and T2r, T4rf and T4r, T6r+ and T6r were degraded by pancreatic deoxyribonuclease. Despite a slow and relatively low extent of cleavage of phosphodiester bonds by this enzyme, the sizes of the resulting fragments, estimated ultracentrifugally, were of the same order as those from the deoxyribonucleic acid of thymus or Escherichia coli. The subsequent cleavage of these fragments by rattlesnake venom phosphodiesterase released mononucleotides and a large proportion of polynucleotide fragments which were fractionated by ion exchange chromatography. Most of the viral 5-hydroxymethyl deoxycytidic acid (dHMP) remained in the polynucleotide fragments. However of the dHMP mononucleotides released, similar distributions of the several types were observed in digests of parental r+ and mutant r strains. The dHMP nucleotides isolated from T2 strains were of two types, monoglucosylated and nonglucosylated, in contrast to the monoglucosylated dHMP nucleotide isolated from T4 strains. Three types were isolated from T6 strains, including similar amounts of a diglucosylated and a nonglucosylated plus a small portion of the monoglucosylated derivative. In addition T6 strains yielded a dinucleotide of dAMP and of diglucosylated dHMP. Spectrophotometric, chromatographic, and electrophoretic properties of the three types of dHMP nucleotides are recorded.

**REFERENCES**

Nucleotides Derived from Enzymatic Digests of Nucleic Acids of T2, T4, and T6 Bacteriophages
Janet Lichtenstein and Seymour S. Cohen


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