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# Attempts to Establish Whether Glucose Is Attached to the Deoxyribonucleic Acid of Certain Bacteriophages Infecting *Bacillus subtilis**

Alvaro H. Alegria† and Fred M. Kahan‡

ABSTRACT: Acid hydrolysates of deoxyribonucleic acid (DNA) from the *Bacillus subtilis* phages PBS2 and SP8 were found by colorimetric analysis to contain, respectively, less than 2.5 and 5.4 moles of glucose per 100 moles of nucleotides. These values are from 4 to 20 times lower than the extents of glucosylation that had previously been reported by others for these DNAs. The physical properties (thermal stability and buoyant density) and base ratios of our samples were however identical with those they had reported. While substantial amounts of glucose could be released by hot acid from intact phage suspensions, it was demonstrated that the glucose-containing material was a polymer that was separable by physical means from the phage DNA. Electrophoresis of nucleotides, released enzymatically from phage DNAs labeled with [1-$^{14}$C]glucose, revealed no components other than the four expected deoxyribonucleotides, nor any discrepancies in relative specific radioactivity that might have indicated the presence of hexoses attached to these nucleotides. Similar analyses of DNAs from phage SP82 and SP8-R, both of which contain 5-hydroxymethyluracil (as does SP8), revealed no significant amount of glucose or other hexoses covalently attached to the DNA. We believe that the glucose associated with these phage DNAs, was contained in contaminating bacterial polysaccharide, probably teichoic acid.

There is abundant evidence that glucose monomers and dimers are covalently linked to the DNA of the T-even bacteriophages of *Escherichia coli* (Lehman and Pratt, 1960; Kuno and Lehman, 1962). There are reports that glucose is also attached to the DNA isolated from two groups of *Bacillis subtilis* phages. These phages resemble the T-even group, in that they contain unusual bases in their DNA. In the case of PBS2, whose DNA contains uracil in place of thymine (Takahaski and Marmur, 1963a), glucosylation of a fraction of the guanine and cytosine residues (20 and 60%, respectively) was demonstrated by these same authors (1963b). Subsequently Rosenberg (1965) reported that glucose was associated with SP8 DNA, in which thymine is replaced by 5-hydroxymethyluracil (Kallen *et al.*, 1962) and that mannose was present in the DNA of SP8*$T_s$, a host range mutant of SP8. In each case Rosenberg found approximately 1 mole of hexose/mole of nucleotide. It was suggested in the above papers that the presence of such substantial quantities of hexose might in part account for the anomalous buoyant densities and thermal stabilities of PBS2 and SP8 DNAs. Erikson and Szybalski (1964) had already demonstrated that glucosylation affected the buoyant density of DNA from the T-even phages, especially in $Cs_2SO_4$ gradients. Furthermore, Langridge and Marmur (1964) observed similar anomalies in the X-ray diffraction patterns of DNA from PBS2 and T-even phages and suggested that they may be related in both cases to glucosylation.

We wished to establish the chemical nature of the linkage of hexoses to the polynucleotide chains in these *B. subtilis* phages, and therefore performed colorimetric, enzymatic, and radiochemical analyses of the DNAs from phage PBS2 and several variants of SP8 in an attempt to confirm the earlier studies. This paper reports our inability to detect by these means any substantial quantity of glucose or other hexoses covalently attached to our DNA preparations. The physical properties of our samples were, however, identical with those reported in prior studies. We will present reasons for believing that contamination of phage particles by bacterial polysaccharides might account for several of the earlier findings that glucose was associated with these phage DNAs.

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† Predoctoral fellow of the Rockefeller Foundation. Present address: Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, Bronx, N. Y. 10461.

‡ Present address: Merck Sharp and Dohme Research Laboratories, Rahway, N. J. 07065.

## Materials and Methods

*Bacteria and Bacteriophages.* The bacteriophages and corresponding bacterial hosts are presented in Table I. *B. subtilis* Marburg (ATCC 6051) is the type strain, a prototroph; *B. subtilis* 168 (Burkholder and Giles, 1947) requires indole or tryptophan for growth and *B. subtilis* SB 19 (Nester and Lederberg, 1961) is a prototroph derived from *B. subtilis* 168.

Phages PBS2, SP8, and SP8-R were obtained from Dr. Julius Marmur. SP8-R, a phage with the same base composition of SP8, grows only in *B. subtilis* 168 or its derivatives and was originally isolated in the laboratory of Dr. W. R. Romig. SP82 was provided by Dr. Donald Green (Green, 1964).

*Reagents.* D-Mannose (Calbiochem, Los Angeles) was recrystallized according to the method of Bates and associates (1942). A 2% solution in water gave $[\alpha]_D^{20}$ +14.4° at equilibrium. Anthrone (9,10-dihydro-9-ketoanthracene) was obtained from Nutritional Biochemicals Corp., Cleveland; the regular Glucostat (glucose oxidase) reagent and crystalline pancreatic DNase I from Worthington Biochemical Corp., Freehold, N. J.; purified snake venom phosphodiesterase from Sigma Chemical Co., St. Louis; and crystalline egg white lysozyme from Calbiochem.

D[1-$^{14}$C]Glucose (3.0 mCi/mmole) was obtained from New England Nuclear Corp., Boston, Mass. Culture media were obtained from Difco, Detroit. All other reagents were of analytical grade.

*Culture Conditions.* The liquid culture media used were: (1) H broth (Chase and Doermann, 1958), supplemented with $CaCl_2$ ($2.5 \times 10^{-3}$ M) and $MnCl_2$ ($10^{-5}$ M); (2) minimal medium (Nomura *et al.*, 1962) containing glucose in place of glycerol. This medium was further supplemented with 1 mg/ml of casein hydrolysate (Casamino Acids, Nutritional Biochemical Corp.).

Phages SP8, SP82, and SP8-R were titrated by standard methods (Adams, 1959) using Hershey's bottom and top agars (Chase and Doermann, 1958). The top agar was supplemented with $MnCl_2$ ($5 \times 10^{-5}$ M). PBS2 titers were determined according to the method of Takahashi (1963).

*Phage Lysates and Purification of the Phages.* Large-scale phage lysates (15 l.) were prepared in carboys mechanically stirred and force aerated at 37°. Foaming was controlled with silicone antifoam (General Electric, Waterford, N. Y.). The bacterial cultures in the exponential phase of growth, with a cell density of $2 \times 10^8$ cells/ml, were infected by the addition of one-fifth volume of a fresh phage lysate containing $10^{10}$ pfu[1]/ml. Large multiplicities were used to achieve simultaneous infection and lysis of most of the cells. Lysis started about 50 min after the addition of phage and was practically complete in about 80 min for all the studied phages. Lysozyme (20 μg/ml) was added afterwards and incubation was continued for 30 min. The lysates were stored at 4°.

After removal of bacterial debris by centrifugation at 5000*g* for 10 min, the phage was sedimented at 16,000*g* for 100 min. The phage pellets were slowly resuspended in 0.1 M potassium phosphate (pH 7.4)–0.01 M $MgCl_2$ during a period of about 12 hr. The turbid suspension was again centrifuged at 5000*g* for 10 min to remove aggregated material and then at 16,000*g* for 100 min to obtain the final phage pellets which were resuspended as before. The volume of the final phage suspension was usually 0.5% that of the original lysate. All the operations were carried out at 4°.

*Phage DNA* was generally obtained by phenol extraction at 24° (Mandell and Hershey, 1960). The phage was lysed prior to the phenol treatment by the addition of 1% sodium dodecyl sulfate (SDS).[1] Virus suspensions containing about 500 μg of phage DNA/ml were extracted three times with two volumes of phenol previously equilibrated against 0.5 M potassium phosphate (pH 7.0). The phenol remaining in the aqueous was eliminated by extraction with ethyl ether free of peroxides or by extensive dialysis against 0.15 M NaCl–0.015 M sodium citrate (pH 7.0).

Native DNA concentrations were determined spectrophotometrically using a molar extinction coefficient per mole of phosphate, $\epsilon_{260}$ (P), of $6.6 \times 10^3$ for the three hydroxymethyluracil-containing DNAs and of $6.9 \times 10^3$ for that of PBS2. Total phosphorus was measured by the method of Chen *et al.* (1956) after ashing with 10% $Mg(NO_3)_2$ in 95% ethanol (Ames and Dubin, 1960). The amount of DNA recovered by the combined detergent and phenol extraction ranged from 90 to 95% of that which could be extracted from the same viral suspension by successively denaturing the phage with cold acid and then digesting out the DNA with DNase (for latter conditions, see under Table V).

*Hydrochloric Acid Hydrolysis.* The DNA was precipitated with two volumes of 95% ethanol, wound on a glass rod, and air dried before it was subject to acid or enzymatic hydrolysis. Hydrolyses of phage DNA or whole phage suspensions were conducted in sealed, glass ampules employing 1 N HCl at 100° for 90 min. For the release of free glucose from bacterial cells the time of hydrolysis was increased to 4 hr.

*Glucose Determination.* The glucose oxidase method (Glucostat, Worthington Biochemical Corp.) was used for the determination of glucose. The method was scaled down for the detection of quantities as small as 0.5 mμmole. The amber color obtained at pH 7.0 (Blecher and Glassman, 1962) was monitored continuously at 450 mμ. The amount of color at any time of the reaction was proportional to the sugar concentration in the range between 1 and 16 mμmoles. At these concentrations maximum color values were obtained by 45 min and the color was stable for about 2 hr.

*Hexoses Determination.* The anthrone procedure as modified by Scott and Melvin (1953) was used in the search for both glucose and mannose, except that 1.0 ml of a 0.2% anthrone reagent in 3.0-ml glass-stoppered tubes and 0.5 ml of the tested solution were used instead

[1] Abbreviations used: SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; dUMP, deoxyuridine 5′-monophosphate HMdUMP, 5-hydroxymethyl-dUMP; dCMP, deoxycytidine 5′-monophosphate; dAMP, deoxyadenosine 5′-monophosphate; GdMP deoxyguanosine 5′-monophosphate; HMU, 5-hydroxymethyluracil; pfu, plaque-forming units.

of the usual volumes. After completion of the reaction the absorption spectrum between 450 and 750 mμ was recorded using a Beckman DK-2 spectrophotometer. The values at 625 mμ (absorption maximum for both glucose and mannose) were linear for both sugars in the concentration range from 25 to 800 mμmoles/ml. The presence of small quantities of hexose in a DNA preparation could be seen as a shoulder at 625 mμ in the peak produced by the DNA deoxyribose (maximum at 565 mμ) (Gary and Klausmeier, 1954) and the amount could be calculated from the difference between the observed value and the value given by calf thymus DNA at the same concentration. T2 DNA, assayed by this method, gave a value of 0.145 mole of glucose/mole of nucleotide (expected value, 0.140; Lehman and Pratt, 1960).

*Labeling of Phage DNA in Media Containing* D[$^{14}$C]-*Glucose.* Radioactive SP8 was prepared according to the following protocol. Minimal medium (1 l.) with 0.6 mg/ml of glucose in place of glycerol, 1 mg/ml of Casamino Acids, and 25 g/ml of each uracil and adenine was inoculated with 1 ml of a culture of *B. subtilis* Marburg grown in the same medium. Maximum $OD^{1\,cm}_{660\,m\mu}$ values under these culture conditions were 0.600 (about $5 \times 10^8$ cells/ml) for *B. subtilis* Marburg and 168 and 0.550 for SB 19, with generation times of about 50 min. After the cells were grown to an $OD^{1\,cm}_{660\,m\mu}$ of 0.300, a 100-ml fraction was taken to be cultured separately and replaced by 100 ml of a fresh lysate of SP8 prepared in the same medium. From the 100-ml cultured part, 10 ml was taken out at the same time, discarded, and replaced by 10 ml of minimal medium free of any supplements. Five minutes after the infection 1.0 and 0.1 ml of a D-[1-$^{14}$C]glucose solution (33 μCi/ml) was added to the infected and uninfected cultures, respectively. Twenty-five minutes later, 100 ml of the infected culture was taken out, 5 ml was separated, and the rest was chilled in iced water after the addition of 15 ml of cold 50% trichloroacetic acid. The whole uninfected culture was treated in the same way. The rest of the infected culture was allowed to lyse normally. After 90 min, lysozyme, to a final concentration of 20 μg/ml, was added and incubation was continued for 30 min at 37°. Purified phage and phage DNA were prepared as described before. The samples removed earlier were used for the determination of the specific radioactivities of the glucose in the culture medium (5-ml sample) and of that incorporated into phage-infected and noninfected cells (95-ml sample plus TCA).

Growth of SP8-R was carried out using the above conditions except that *B. subtilis* SB 19 was used as a host. In the case of SP82, *B. subtilis* 168 (trp 2) was the host, requiring the addition of tryptophan (25 μg/ml) to the culture medium. Other conditions were the same except for a higher amount of radioactive glucose (50 μCi). PBS2 phage was grown in *B. subtilis* Marburg, also using 50 μCi of radioactive glucose. However the preparation of this phage was carried out in the absence of both uracil and adenine, thus accounting for the higher radioacitivity of the DNA in this case (Table III).

DNA radioactivities were determined using the same solutions utilized for the spectrophotometric determinations to avoid errors due to the high viscosity of the preparations. The DNA solution taken from the cuvet was precipitated with 5% cold TCA, collected on a Millipore filter, and washed with 5% TCA and 95% ethanol. After drying, the filter was placed in a glass vial with 18 ml of toluene containing 5 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-2(4-methyl-5 phenyloxazolyl)-benzene per l. and counted in a Packard Tri-Carb scintillation counter with an efficiency of 60%.

*Enzymatic Hydrolysis of DNA.* DNA (1–2 μmoles) previously precipitated by ethanol was resuspended in 0.4 ml of 0.01 M Tris–0.005 M $MgCl_2$ (pH 7.4) plus 100 μg/ml of bovine serum albumin. Degradation to mononucleotides was effected by the successive action of pancreatic DNAse I and purified snake venom phosphodiesterase, essentially as described by Cohn (1957).

Mononucleotide concentrations were determined spectrophotometrically both in 0.01 N HCl and in 0.05 M potassium phosphate (pH 7.0). Radioactivities were determined with the same nucleotide solutions (1.0 ml) in 18 ml of Bray's (1960) solution with a counting efficiency of 55%.

*Paper Electrophoresis and Chromatography.* Whatman No. 3MM was used in both procedures. Electrophoretic separation of DNA constituents was carried out in a Savant FP-22 apparatus, using gradients of 30 V/cm for 3 hr and 0.05 M ammonium formate at a pH of 3.5. Paper strips were scanned for radioactivity in a Vanguard 880 apparatus.

The descending technique was used throughout for the chromatographic separation of sugars. Separation after desalting was carried out with ethyl acetate–pyridine–water (2:1:2, v/v) (Jermyn and Isherwood, 1949). In this system glucose has an $R_F$ of 0.28 and mannose 0.32. Salts were eliminated from culture media aliquots or from acid hydrolysates prior to paper chromatography by passing the solutions (5 ml) first through a Dowex 50-X8 ($H^+$) column (200–400 mesh, $0.4 \times 10$ cm) and then through an Amberlite IR-45 ($OH^-$) column (20–50 mesh, $0.4 \times 10$ cm). Sugar spots were developed on paper chromatograms with the aniline phosphate reagent (Bryson and Mitchell, 1951).

## Results

It is shown in Table I that the host ranges of our PBS2 and SP8 preparations, as well as their DNA buoyant densities and thermal stabilities, are similar to earlier descriptions of these properties. Also presented in Table I are the properties of the 5-hydroxymethyluracil containing phages SP82 and SP8-R, which with the exception of host range, approximate closely those of SP8. Rosenberg's (1965) finding that the DNA of SP8*$T_s$ contains mannose prompted the study of SP82 and SP8-R, both with a host range similar to that of SP8*$T_s$.

*Analyses of Purified DNA.* COLORIMETRIC ASSAYS FOR HEXOSES. Our glucose oxidase analyses of DNA HCl hydrolysates, presented in Table II, show that the DNA from PBS2 had a low though variable glucose content that never exceeded 20% of the level reported by Takahashi and Marmur (1963b) in their preparations. DNA

TABLE I: Host Range and Physical Properties of the *B. subtilis* Phages Analyzed.

| Phage | Host Range[a] | Unusual Base[b] | Physical Properties of Phage DNA[c] | | | |
|---|---|---|---|---|---|---|
| | | | Buoyant Density in CsCl (g/cc) | | Thermal Denaturation Temp (°C)[d] | |
| | | | Quoted | Found | Quoted | Found |
| PBS2[e] | Marburg, 168, SB 19 | Uracil | 1.722[f] | 1.722 | 76.5[f] | 76.5 |
| SP8[g] | Marburg | 5-Hydroxymethyluracil | 1.743[h] | | | |
| | | | 1.746[i] | 1.742 | 77.8[i] | 77.5 |
| | | | 1.744[j] | | 76.5[j] | |
| SP82[k] | 168, SB 19 | 5-Hydroxymethyluracil | 1.742[k] | 1.742 | 75.0[k] | 77.8 |
| SP8-R | 168, SB 19 | 5-Hydroxymethyluracil | | | | 77.5 |

[a] The ability of phage lysates to produce plaques on agar plates bearing bacterial lawns of *B. subtilis* strains Marburg, 168 and SB 19 was tested. Those strains which permitted plaque formation are listed. [b] The unusual base replaces thymine in each case. [c] The physical properties of the DNA from a given phage were found to be independent of the host in which the phage was produced. [d] That temperature at which half of the maximum thermally induced hyperchromicity is observed when native DNA is heated in 0.15 M NaCl–0.015 M trisodium citrate. [e] Takahashi (1963). [f] Takahashi and Marmur (1963b). [g] Romig and Brodetsky (1961). [h] Marmur and Cordes (1963). [i] Rosenberg (1965). [j] Kallen *et al.* (1962). [k] Green (1964).

TABLE II: Free Glucose Liberated by Acid Hydrolysis of Phage DNA.[a]

| Phage | Host | Amt of DNA Hydrolyzed (mμmoles of nucleotides | D-Glucose Liberated (mμmoles) | Mole % of Glucosylation (moles of glucose/mole of nucleotide) × 100 | |
|---|---|---|---|---|---|
| | | | | Quoted | Found |
| PBS2 | Marburg[e] | 172 | 2.5 | 12[b] | 1.4 |
| | Marburg | 139 | 1.0 | | 0.7 |
| | SB 19[e] | 116 | 2.3 | | 2.0 |
| | SB 19 | 200 | 0.0[c] | | 0.0 |
| SP8 | Marburg[f] | 105 | 5.7 | 98[d] | 5.4 |
| | Marburg[e] | 141 | 1.8 | | 1.3 |
| SP82 | 168 | 222 | 0.6 | | 0.3 |
| | 168 | 190 | 0.8 | | 0.4 |
| SP8-R | SB 19[f] | 148 | 1.3 | | 0.9 |
| | SB 19 | 191 | 3.6 | | 1.9 |

[a] The DNA of phage particles purified from two independent lysates of each phage–host combination, was hydrolyzed in HCl and assayed for free D-glucose as described in Materials and Methods. In no DNA preparation could free glucose be detected prior to acid hydrolysis. [b] Takahashi and Marmur (1963b). The strain of *B. subtilis* they used was not specified. [c] The assay could detect at least 0.5 mμmole of D-glucose/sample. [d] Rosenberg (1965). [e] In Table IV of this paper, an analysis is presented of the glucose associated with the phage suspensions which provided these samples of DNA. [f] The hexose content of these DNA preparations was determined by the anthrone method (see text).

prepared from this phage (grown on strain Marburg) using SDS as the exclusive phage disruptive agent was also examined. This procedure had been employed previously by Kuno and Lehman (1962) to isolate the glucosylated DNA from T2 phage. HCl hydrolysis of 3.0 μmoles of DNA nucleotides from such a preparation yielded only 22 mμmoles of free glucose (0.73% glucosylation of the nucleotides), confirming the initial low results. The amounts of glucose liberated from SP8 DNA on acid hydrolysis (Table II) were also variable, but again represented at most 5% of the level of glucosylation reported by Rosenberg with this phage. Similarly

TABLE III: Analysis of Mononucleotides from [1-$^{14}$C]Glucose-Labeled Phage DNA.

| Phage–Host Combination | Sp Radioactivity (cpm/μmole × $10^{-3}$) | | | Amount of DNA Added to Digest (mμmoles) | Nucleotide | Amount of Nucleotide Recovd[b] (mμmoles) |
|---|---|---|---|---|---|---|
| | Exogenous Glucose[a] | Phage DNA | Nucleotide | | | |
| PBS2–Marburg | 30.0 | 26.0 | 19.5 | 1950 | dUMP | 710 |
| | | | 20.4 | | dCMP | 260 |
| | | | 30.0 | | dAMP | 675 |
| | | | 26.2 | | dGMP | 258 |
| | | | | | | Sum 1903 |
| SP8–Marburg | 21.8 | 2.4 | 2.27 | 1200 | HMdUMP | 330 |
| | | | 1.87 | | dCMP | 252 |
| | | | 2.57 | | dAMP | 335 |
| | | | 1.73 | | dGMP | 256 |
| | | | | | | Sum 1173 |
| SP8-R–SB 19 | 19.6 | 0.93 | 0.87 | 1000 | HMdUMP | 272 |
| | | | 0.68 | | dCMP | 196 |
| | | | 1.01 | | dAMP | 281 |
| | | | 0.73 | | dGMP | 208 |
| | | | | | | Sum 947 |
| SP82–168 | 30.0 | 4.0 | 4.46 | 1300 | HMdUMP | 350 |
| | | | 3.32 | | dCMP | 266 |
| | | | 3.78 | | dAMP | 372 |
| | | | 3.48 | | dGMP | 268 |
| | | | | | | Sum 1256 |

[a] The specific radioactivities of glucose were determined on 5-ml samples removed 30 min after the addition of the phage. After rapid chilling to 0° the cells were sedimented by centrifugation. The supernatants were desalted as described in Materials and Methods and then subjected to paper chromatography in ethyl acetate–pyridine–water. The glucose-containing region was identified by spraying one half of the strip with aniline phosphate. The colored spot encompassed the only major radioactive peak observed in a scanning of the paper. This region was eluted from the unstained half with water and the glucose concentration was determined by the glucose oxidase method. Radioactivities were determined in Bray's (1960) solution with a counting efficiency of 55%. [b] Elution was carried out with water. A paper blank correction was carried out in each case.

ow levels of glucose were found associated with the DNAs of phages SP82 and SP8-R.

To determine whether other hexoses in addition to glucose might be associated with certain phage DNAs, samples of DNA were treated directly with the anthrone reagent (Scott and Melvin, 1953) following the example of Rosenberg (1965) who by this procedure had detected D-mannose in the DNA of SP8*$T_8$. By this method the presence of 5.9 and 0.6 mole % hexoses in preparations of SP8 and SP8-R DNA, respectively, was found.

NATURE OF THE ASSOCIATION OF GLUCOSE WITH SP8 DNA. To approach the question of whether or not the glucose-containing material found in several of our DNA preparations was in fact covalently attached to the nucleic acid, we carried out the following fractionation of the most extensively glucosylated sample, that from SP8 DNA bearing 5.4 mole % glucose (Table II). To a solution containing 2.8 moles of DNA nucleotides, TCA was added to a final concentration of 5% and the precipitate that formed after 10 min at 0° was sedimented and redispersed in 0.5 ml of water. This suspension was extracted twice with equal volumes of ether to remove traces of TCA, and then received 10 moles of potassium phosphate (pH 7.0), 5 μmoles of $MgCl_2$, and 50 μg of pancreatic DNase I. The resulting clear solution was examined spectrophotometrically and assayed for free glucose, both before and after hydrolysis with HCl under the standard conditions. Less than 3 mμmoles of glucose/μmole of nucleotide was detected; however, 96% of the nucleotides, introduced as DNA at the time of precipitation, was recovered. Meanwhile, the supernatant remaining after the precipitation of the DNA was also freed of TCA by extraction with ether and analyzed for acid-liberatable glucose and nucleotide content as above. Less than 0.08 μmole of nucleotide was detected; however, 140 mμmoles of free glucose was liberated by acid hydrolysis.

ANALYSIS OF DNA FROM PHAGE GROWN IN THE PRES-

ENCE OF LABELED GLUCOSE. Enzymatic hydrolysates of DNA from phage that had been grown in the presence of [1-$^{14}$C]glucose were examined for evidence of radioactive substituents attached to the nucleotides. [1-$^{14}$C]-Glucose was chosen to minimize labeling of the deoxyribose moiety. The conditions employed to label phage lysates are described under Materials and Methods. Phages were purified and DNA was isolated as for previous experiments.

Phage DNAs, in amounts indicated in Table III, were degraded enzymatically to mononucleotides. The enzymatic digest was then applied directly to chromatography paper and subjected to electrophoresis under the conditions employed to resolve the four normal deoxyribonucleotides (see Materials and Methods). In each case, four bands were observed under ultraviolet light and a subsequent scan of the paper strip revealed significant radioactive peaks only under the ultraviolet-absorbing regions. No more than 3% of the input radioactivity could be detected at the origin of the electropherogram. Each of the four bands was then bisected into a slow- and fast-running half and these were separately eluted. We thereby hoped to detect, as variations in specific radioactivity between the two halves of a given band, any component whose electrophoretic mobility might differ slightly from the parent nucleotide as a consequence of hexosylation. The ratios of radioactivity to ultraviolet absorbance in any pair of eluates, were in fact found to differ by no more than 3%.

The total radioactivity and nucleotide content of the four bands from each electropherogram are presented in Table III. Depending on the source of the phage DNA, the identity of the fastest migrating band was established as either dUMP or 5-hydroxymethyl-dUMP (HMdUMP) from their ultraviolet spectral values (see Kallen *et al.*, 1962). The proportion of (dCMP + dGMP) observed in these experiments was, in the case of PBS2 DNA, 27 mole % of total nucleotides, and for the HMdUMP-containing phages averaged 43 ± 1 mole %. These base ratios compare well with the published values of 28 and 43.1% for PBS2 DNA (Takahashi and Marmur, 1963b) and SP8 DNA (Kallen *et al.*, 1962), respectively. The total amount of nucleotides recovered in each experiment was, as judged by ultraviolet absorbance, within 4% of the quantity of DNA nucleotides (in the form of native DNA) added to the digest. The total amounts of radioactivity eluted from the ultraviolet-absorbing bands corresponded to between 96 and 98% of the introduced radioactivity. By these criteria, it appears unlikely that selective losses of any component of the original DNA preparations have occurred during the analysis.

To assess further the possibility of glucosylation in the case of PBS2, 188 mμmoles of the dCMP fraction eluted from the electropherogram was combined with 50 mμmoles of cold glucose and following lyophylization, the mixture was treated with 1 N HCl at 100° for 2 hr. Separation of the glucose from other hydrolysis products was carried out first by paper chromatography in isopropyl alcohol–water (80:20, v/v) and then by paper electrophoresis. No radioactivity could be found in the glucose spot which remained at the origin.

Finally, the ability of phage-infected cells to incorporate labeled glucose into high molecular weight cell components was tested. During the preparation of radioactive SP8 and SP8-R phages, both infected and noninfected *B. subtillis* Marburg and SB19 cells, respectively, were also obtained (see Materials and Methods). One extraction with 7% TCA and two with 75% ethanol were then carried out (Hash, 1962) to eliminate low molecular weight components. After HCl hydrolysis of the whole cells and separation of the sugars by paper chromatography, the specific radioactivities of the glucose were determined as described under Table III. The results, compared with those of the glucose in the respective culture medium, are presented in Table IV. While

TABLE IV: Specific Radioactivity of Glucose in Hydrolysates of Infected and Uninfected *B. subtilis* Cells.

| | Sp Radioact. (cpm/μmole) | |
|---|---|---|
| Cells Source | [1-$^{14}$C]Glucose in Culture Medium | Glucose Hydrolyzed from Cells |
| *B. subtilis* Marburg | 22,000 | 21,400 |
| *B. subtilis* + Marburg SP8 | 21,800 | 10,900 |
| *B. subtilis* SB19 | 22,700 | 16,700 |
| *B. subtilis* + SP8-R SB19 | 19,600 | 7,100 |

noninfected cells showed a rapid incorporation of the label, in phage-infected cells decreased but still substantial incorporation could be observed. An incidental observation made in these experiments was that free D-mannose, the hexose claimed by Rosenberg to be present in the DNA of SP8*$T_s$, is not present in hydrolysates of the cells of this phage's host, *B. subtilis* SB19.

*Glucose Associated with Phage Particles.* Since our phage DNA preparations had been revealed by the above analyses to be relatively free of glucose, suspensions of intact phage particles were next examined for their glucose content. Acid hydrolysates of PBS2 suspension (Table V) contained from three to seven times as much free glucose as could be liberated from the DNA isolated from equivalent quantities of phage suspension (*cf.* Table III). Suspensions of SP8 particles contained only 50% more acid-liberatable glucose than the DNA they yielded. The nature of this glucose-containing material was then studied by a fractionation procedure similar to that used in this work to demonstrate that the glucose associated with SP8 DNA was physically separated from it.

Phage particles were first precipitated with TCA; approximately one-fourth of the original glucose-containing material remained acid soluble. The DNA contained in the acid-precipitated and -denatured phage was then digested out of the disrupted particles under mild, enzymatic conditions described in the legend of Table V,

TABLE V: Acid-Liberated Glucose in Whole and Fractionated Phage Suspensions.[a]

| Phage | Host | Amount of DNA in Phage Samples (μmoles) | Acid-Liberatable Glucose: Whole Phage: mμmoles | Acid-Liberatable Glucose: Whole Phage: (moles of glucose/mole of nucleotide) × 100 | Phage Fractions: Acid Soluble | Phage Fractions: Nucleotides | Phage Fractions: Phage "Protein" |
|---|---|---|---|---|---|---|---|
| PBS2 | Marburg | 4.8 | 451 | 9.4 | 73 | <5 | 354 |
| PBS2 | SB 19 | 7.2 | 437 | 5.8 | 72 | <5 | 375 |
| SP8 | Marburg | 9.8 | 185 | 1.9 | 45 | <5 | 150 |

[a] Those preparations of phage from which the DNA samples designated *e* in Table II had been isolated, were assayed for glucose using glucose oxidase before and after treatment with 1 N HCl at 100° for 90 min. No glucose was detected before hydrolysis; the amount liberated by the acid treatment is recorded under *whole phage*. To obtain the three *phage fractions* described, TCA to a final concentration of 5% was added to 5-ml samples of phage suspension and the precipitate that formed after 10 min at 0° was sedimented. TCA was eliminated from both the supernatant (*acid-soluble fraction*) and the phage precipitate by multiple extraction with peroxide-free ethyl ether. The denatured phage was resuspended in 2 ml of 0.05 M potassium phosphate (pH 7.0)–0.01 M $MgCl_2$, containing 50 μg/ml of pancreatic DNase I. After this mixture was incubated at 37° for 3 hr, insoluble material was sedimented and the optical density at 260 mμ was measured to establish the total DNA content of the phage sample analyzed ($E_{mM}$ 9.6 assumed). TCA was again added to a final concentration of 5%, and the precipitate that formed was sedimented. The precipitate was resuspended in water, and both it (*the phage "protein"*) and the supernatant (*nucleotides*) were first extracted with ether, then assayed for ultraviolet absorbance and acid-liberatable glucose. A minimum of 5 mμmoles of glucose could have been detected in the *nucleotide* fractions.

and TCA was again added to separate the acid-soluble nucleotides from the phage "protein" which was still precipitable. Glucose could not be detected in acid hydrolysates of the nucleotide fraction, even though it contained more than 95% of the input ultraviolet absorbancy. Most of the glucose originally associated with the acid-precipitated phages could, however, be liberated from the nucleic acid-free phage "protein."

## Discussion

Previous reports that the DNAs of phages PBS2 and SP8 were extensively glucosylated could not be confirmed by us, even though our preparations of DNA had all other properties such as base compositions, buoyant densities, and thermal stabilities identical with those reported by other laboratories.

Attempts were first made to verify reports that free glucose was liberated from phage DNA on treatment with hot acid. Our glucose oxidase determinations (Table III) indicated, however, a low glucose content in all the DNA preparations examined, much lower in the case of PBS2 and SP8 than the values previously reported. A more detailed study made in the case of SP8 DNA showed that the glucose-containing material accompanying this DNA preparation was either separate from the DNA or attached to it by a remarkably labile linkage. The quantity of total hexoses in the DNAs from SP8 and SP8-R, estimated with the anthrone reagent, was approximately equal to the level of glucose *per se* assayed by use of the specific glucose oxidase system. Thus, additional hexoses such as mannose, which Rosenberg had isolated from the DNA of SP8*$T_8$, appear to be absent from these two DNAs.

Although our methods for the preparation of the phage DNAs and for the detection of glucose are similar to those used by other authors, we considered the possibility that glucose might originally be present in these DNAs, but could experience a chemical rearrangement during the isolation or acid treatment of the nucleic acid to yield products which might fail to react with glucose oxidase and anthrone. This situation was conceivable if, for example, the sugar had been attached to an amino group on the bases. Such glucosylamines are known to undergo readily an Amadori rearrangement (Hodge, 1955) to form ketosamines. PBS2 DNA lacks a hydroxymethyl group, analog of that of the T-even phages, for the attachment of glucose and since both its cytosine and guanine residues were previously reported to be glucosylated (Takahashi and Marmur, 1963b), the amino groups in these bases would be likely candidates for substitution. The possibility of such a transformation was tested by examining enzymatic hydrolysates of DNA from phages that had been grown in cells exposed to [1-$^{14}$C]glucose. It is noteworthy that every DNA sample could be completely digested to mononucleotides by a combination of pancreatic DNase I and snake venom phosphodiesterase, since these enzymes were found by Sinsheimer (1954) and Volkin (1954) in their analyses of glucosylated T-even phages

DNA, to be incapable of hydrolyzing most oligonucleotide sequences containing glucosylated bases.

In the search for specifically substituted mononucleotides, it seemed reasonable to compare the specific radioactivities of each pair of pyrimidine or purine nucleotides, inasmuch as each pair probably shares a common ribonucleotide precursor, uridylic or inosinic acid, respectively (Magasanik, 1962). An estimate of the amount of substitution could then be made by assuming that any glucose linked to phage DNA would have a specific radioactivity close to the one in the culture medium. Such an assumption receives some support from the finding that the specific radioactivity of glucose isolated from bacterial cells infected with phages SP8 or SP8-R was still a substantial fraction of that of the exogeneous sugar. The decrease observed could be largely explained by the lack of division and diminished cell wall synthesis that occur after phage infection.

In the case of the nucleotides composing PBS2 DNA (Table III) the specific radioactivities of the dCMP and dUMP regions were, in fact, almost identical and no evidence was found for the 60% glycosylation of the dCMP fraction claimed by Takahashi and Marmur (1963b). In the case of the HMU phages the differences observed between the two pyrimidine nucleotides account for at most 3% glucosylation. Larger differences in specific radioactivity were observed between dAMP and dGMP in the case of PBS2, giving values of glycosylation of about 10% for dAMP, in contrast to the earlier claim (Takahashi and Marmur, 1963b) that dGMP was glucosylated. Although such differences appear to be less significant in the case of the HMU phages, we have no explanation for the fact that in all cases dAMP was between 1.1 and 1.5 times more radioactive than dGMP.

Although our DNA preparations were relatively free of glucose, substantial amounts of the sugar could be liberated from intact phage preparations by acid hydrolysis. In some cases the ratio of glucose liberated to DNA contained in these preparations approached that reported by the other laboratories in their purified nucleic acid preparations. It was therefore conceivable that the DNA we prepared either by phenol or detergent lysis of phage particles represented only a fraction of their total contents and that there might remain, unextracted, a fraction of DNA that was extensively glucosylated. Yet we were able to show that acid-denatured phage, exposed to DNase, yielded at least 95% of its nucleic acid in an acid-soluble form while retaining in an acid-precipitable condition most of the material from which glucose could be liberated by hot acid. Such glucose must therefore be present in a polymer which is either completely independent of DNA, or attached to less than 5% of its constituent nucleotides.

Although our conclusions regarding PBS2 DNA are irreconcilable with the original observations of Takahashi and Marmur (1963b) that glucose is attached covalently to guanine and cytosine residues, they are in accord with the results of a reinvestigation of this problem by Ulbricht *et al.* (1966) that appeared following the preliminary presentation of the work contained in this paper (Cassidy *et al.*, 1965). Ulbricht *et al.* find that the dCMP fraction isolated from PBS2 DNA prepared in Takahashi's laboratory is now free of glucose; but they offer no alternative explanation for the detailed enzymatic and chromatographic evidence that Takahashi and Marmur had obtained regarding the original glycosylated material. In contrasting our findings with Rosenberg's conclusion that SP8 DNA is extensively glucosylated, it should be noted that he provided no chemical evidence, comparable to that of Takahashi and Marmur, to prove that these hexoses were in fact covalently attached to the DNA chain. Rather, he demonstrated that the buoyant densities in CsCl of DNA and of the material yielding hexoses were identical. Yet at least two cellular polysaccharides (glycogen and teichoic acid) have been shown to accompany DNA preparations from liver cells (Martínez Segovia *et al.*, 1965) and *B. subtilis* (Young and Jackson, 1966), respectively, and to have buoyant densities in CsCl overlapping those of the DNAs. The possibility that the glucose observed by Rosenberg in SP8 DNA was derived from polysaccharides originally contaminating the phage, must be considered in view of the high polysaccharide content of *B. subtilis* (7% of the cell dry weight as reducing sugar; Salton, 1960). Furthermore, about 60% of the dry weight of *B. subtilis* walls consists of teichoic acid extensively substituted with glucose monomers that may be readily liberated as free glucose, by treatment with hot acid (Armstrong *et al.*, 1960). Our observation that a fraction of the glucose-containing material in phage particles (and all of the glucose-containing material occasionally found in DNA preparations) was soluble in cold TCA may be related to the similar, limited solubility in cold acid of the teichoic acid fragments found by Armstrong *et al.* in whole *B. subtilis*.

Such an explanation cannot, however, be suggested for the presence of mannose in SP8*$T_s$, since we were able to establish that its host, *B. subtilis* SB 19, is devoid of any polysaccharides yielding mannose on acid hydrolysis. Further experiments with this particular phage would be necessary to establish the nature of the mannose-containing material. In any case, the amounts of sugar reported for both SP8 and SP8*$T_s$, are so large that it would be necessary to invoke very extensive contaminations indeed to account for such results.

We have also examined the physical properties of synthetic polynucleotides containing uracil or 5-hydroxymethyluracil. They were prepared in reaction mixtures containing DNA polymerase, either SP8 or PBS2 DNA as primers, and a mixture of dATP, dGTP, dCTP, and either HMdUTP or dUTP. The products had buoyant densities and thermal stabilities identical with their primers (Cassidy *et al.*, 1965). Since these polynucleotides could not conceivably contain hexoses, we consider confirmed our conclusion, from the admittedly negative results contained in this paper, that the anomalous physical properties of these phage DNAs results from some influence of the unusual bases themselves. Indeed it is surprising that those DNAs which are claimed to have covalently attached hexoses, nevertheless have physical properties so similar to our authentically hexose-free products.

It must finally be mentioned that glucose has also been shown to be absent from the DNA of two other 5-hydroxymethyluracil-containing *B. subtilis* phages, $\phi$e (Roscoe and Tucker, 1966) and SPO-1 (Okubo *et al.*, 1964), although these DNAs present anomalous physical properties similar to those of SP8.

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