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Studies on Acid Deoxyribonuclease. V. The Oligonucleotides Obtained from Deoxyribonucleic Acid and Their 3′-Phosphate Termini*

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ABSTRACT: Deoxyribonucleic acid (DNA) samples from different sources have been digested with spleen acid deoxyribonuclease. An initial fast phase followed by a terminal very slow one can be recognized in both the hyperchromic shift and the acid-soluble oligonucleotide release taking place during the digestion. DNase digests in the early slow phase are composed of oligonucleotides having an average size of 10–12. Over 65% of the digest is made up of fragments of a size larger than 7, which cannot be resolved by DEAE-cellulose–urea columns. The 3′-phosphate-terminal nucleotides have been determined after digestion with spleen acid phosphomonoesterases I and II and spleen exonuclease, using several different techniques. Deoxyguanylic and deoxyadenylic acids, in about equal amounts, form about 80% of the 3′-phosphate termini; thymidilic and deoxycytidylic acids form 10 to 15 and 5 to 7% of the 3′-phosphate ends, respectively. Various kinds of artifacts explaining the widely different results reported by previous authors have been investigated.

Spleen acid deoxyribonuclease (deoxyribonucleate 3′-oligonucleotidohydrolase (EC 3.1.4.6): deoxyribonuclease II) has been the object of extensive investigations in this laboratory over the past few years. These have shown that the enzyme is able to split simultaneously both DNA strands at the same level (Bernardi and Sadron, 1961, 1964a,b; Mac Hattie *et al.*, 1963) and is competitively inhibited by tRNA, rRNA, and certain synthetic polyribonucleotides (Bernardi, 1964; Jacquemin-Sablon *et al.*, 1964). Spleen acid DNase has been isolated as a homogeneous protein (Bernardi *et al.*, 1963, 1966; Bernardi and Griffé, 1964), characterized in its physical and chemical properties (Bernardi *et al.*, 1965), and shown to be a dimeric protein molecule (Bernardi, 1965). The enzyme can split very slowly bis(*p*-nitrophenyl)phosphate and the *p*-nitrophenyl esters of deoxyribonucleoside 3′-phosphates (Bernardi and Griffé, 1964), but not those of the 5′ derivatives (Bernardi, 1966); in spite of this "phosphodiesterase" activity, the enzyme has no exonuclease activity as first shown by Bernardi and Griffé (1964) and later confirmed by the present work. Acid DNase is a very widely distributed lysosomal enzyme, probably present in all the cells of eukariotic organisms (Cordonnier and Bernardi, 1965; C. Cordonnier and G. Bernardi, to be published).

The specificity of spleen acid deoxyribonuclease has been investigated in several laboratories (Privat de Garilhe and Laskowski, 1954; Laurila and Laskowski, 1957; Koerner and Sinsheimer, 1957; Vanecko and Laskowski, 1961, 1962; Doskocil and Sorm, 1961a,b, 1962). However, in spite of the remarkable efforts devoted to solve this problem, no clear picture of the basic properties of the digest, such as average size and terminal nucleotides, has emerged so far.

In view of this, we decided to investigate this problem thinking that we were in a better position than previous authors for the following reasons: (a) the availability of homogeneous spleen acid DNase preparations (free of exonuclease activity) having a specific activity about three times higher than the best preparations previously used by other workers (Bernardi and Griffé, 1964; Bernardi *et al.*, 1965, 1966); (b) the availability of three

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TABLE I: DNase Digestions

DNA Digest	Solvent (M)	Temp (°C)	DNA Concn (A_{260})	Enzyme Concn (units/ml)	Enzyme Prepn	Digestion Time (hr)	Hyperchromic Shift (260 mμ) (%)	Acid-Soluble Oligonucleotides (260 mμ) (%)
				Calf Thymus[a]				
A	SAB	25	8.88	0.047	HS 11	16	32.2	41.0
B	NH_4Ac (0.05), pH 5.0	37	9.70	0.124	HS 11	16	35.0	35.0
C	SAB	25	7.37	0.19	HS 11	1.5	30.5	
Ca	SAB	37	7.37	0.19	HS 11	+14[b]	35.5	
D	SAB	25	8.13	0.7 + 0.23[c]	HS 17–17	5	33.7	48.0
Da	SAB	25	8.13	0.7 + 0.23[c]	HS 17–18	+14[b]	36.1	
Db	SAB	37	8.13	0.37	HS 17–18	8[d]	34.0	
				Chicken Erythrocytes				
	SAB	37	14.74	0.14	HS 10	2	30.6	33.3
				H. influenzae				
	SAB	37	1.94	0.07	HS 10	4	36.0	34.3
				E. coli[e]				
I	NaAc (0.15), pH 5.5	37	6.30	0.14	HS 10	5.5	27.8	33.7
II	NaAc (0.15), pH 5.0	37	6.33	0.10	HS 10	5	27.8	34.2
III	NaAc (0.15), pH 5.5	37	5.72	0.042	HS 10	2	31.2	37.6

[a] All digestions were done on preparation P 64. [b] These figures indicate the times of further digestion of samples C and D, respectively. [c] Further addition of enzyme was done after 225-min digestion (see Figure 2). [d] This figure indicates the time of further digestion of an aliquot of digest D. [e] Samples I and II come from the same *E. coli* DNA preparation.

other enzymes which were purified from spleen for this study, two acid phosphomonoesterases (Chersi *et al.*, 1966; Bernardi, G., Chersi, A., and Bernardi, A., paper to be published) and an exonuclease (Bernardi and Bernardi, 1966, 1968); and (c) the existence of chromatographic techniques which had not been available to previous authors. These include the DEAE-cellulose–urea chromatography of Tomlinson and Tener (1963), and the chromatography on polyacrylamide gel columns of nucleotides and nucleosides (Carrara and Bernardi, 1968). In the course of this work we have studied in some detail the conditions under which enzymatic digestions, analyses, and concentrating procedures could be carried out. In so doing we have discovered pitfalls and hitherto unsuspected artifacts, which may explain the widely different results obtained by previous investigators. Because of the practical importance of these findings, sufficient details are given in the tables to enable the reader to follow the history of a given DNA digest through the many steps leading to the determination of size and terminal nucleotides.

Material and Methods

DNA preparations were obtained from calf thymus and chicken erythrocytes, *Escherichia coli*, and *Haemophylus influenzae* by using a detergent procedure (see Bernardi and Sadron, 1964b).

Enzymatic Digestions. A very wide variety of experimental conditions (enzyme concentrations and preparations, digestion times, and solvents) were used in the enzymatic digestions. These were carried out at 37° or at room temperature. The substrate concentration was close to 400 μg/ml of DNA or DNA oligonucleotides, except where otherwise stated. Enzyme units were defined elsewhere (see Bernardi *et al.*, 1966, for acid DNase; Chersi *et al.*, 1966, for acid phosphomonoesterases; Bernardi and Bernardi, 1968, for exonuclease).

Acid DNase. Digestions were carried out with four different enzyme preparations (HS 10, 11, 13, and 17–18) obtained from hog spleen according to the method of Bernardi and Griffé (1964), as modified by Bernardi *et al.* (1966). The buffers which were most widely used in the digestion mixtures were 0.15 M sodium acetate–0.01 M EDTA (pH 5.0) (standard acetate buffer)[1] and 0.05 M ammonium acetate (pH 5.5). The enzyme concentra-

[1] Abbreviation used: SAB, standard acetate buffer, is 0.15 M acetate buffer–0.01 M EDTA (pH 5.0).

tions used varied from about 0.05 to 1 unit/ml of incubation mixture (see Table I). Digestions were followed by measuring the hyperchromic shift at 260 mμ and the liberation of acid-soluble oligonucleotides. These were determined as described by Bernardi and Griffé (1964); values were corrected for dilution with enzyme and perchloric acid. Sometimes, a second addition of an equal amount of enzyme was made after the slow phase of the digestion had been reached (see below).

The enzyme inactivation was done by emulsifying the digest with chloroform–isoamyl alcohol (5:1; Sevag, 1934). The emulsion was centrifuged and the top layer was pipetted and dried *in vacuo* in a rotary evaporator at 25–30°; the digest was then dissolved in distilled water, and dried once more. The digest was either redissolved in distilled water and adjusted with acetic acid to pH 5.0–5.5 (if the original solvent was SAB) or in 0.05 M ammonium acetate (pH 5.0–5.5) (if this was the original solvent), and used for the phosphomonoesterase digestion.

Acid Phosphomonoesterases. Acid DNase digests in the sodium or ammonium acetate buffers mentioned above were digested with two different acid spleen phosphomonoesterases. Phosphomonoesterase I is the enzyme described by Chersi *et al.* (1966), which is identical in all its properties with prostatic acid phosphomonoesterase; phosphomonoesterase II is a different enzyme which has also been prepared in this laboratory (G. Bernardi, A. Chersi, and A. Bernardi, paper to be published).

The enzyme concentrations used varied from 0.004 to 0.4 unit per ml of incubation mixture (see Tables III and IV). The lowest concentration of enzyme used was sufficient to release completely in 1 hr at 37° phosphate from 10^{-4} M *p*-nitrophenyl phosphate (a concentration equimolar with that of terminal phosphate in the DNase digest). The time course of the reaction was followed by determining the ratio of total phosphate in the digest to the inorganic phosphate released by the enzyme and verifying that a saturation level had been reached. The inactivation of the enzyme, and the concentration and redissolution of the digest were done as already described above.

Exonuclease. Digestions of the dephosphorylated DNase digests were done using an enzyme preparation obtained from hog spleen according to Bernardi and Bernardi (1966, 1968) in the acetate buffers mentioned above. The concentrations varied from 0.7 to 1.5 units per ml, in excess of the amount required for a complete digestion. The time course of the reaction was followed by determining the hyperchromic shift which takes place during the reaction. That the digestion was complete was checked by verifying that nucleosides and mononucleotides only were present in the final digest. This was done by paper chromatography and DEAE-cellulose chromatography, as described below. The digests were concentrated and dissolved in distilled water if they were to be loaded on Bio-Gel P-2 column or paper; they were diluted ten times and adjusted to pH 8–9 with NH_4OH if they were to be loaded on DEAE-cellulose columns. Figure 1 summarizes the sequence of enzymatic digestions used in this work.

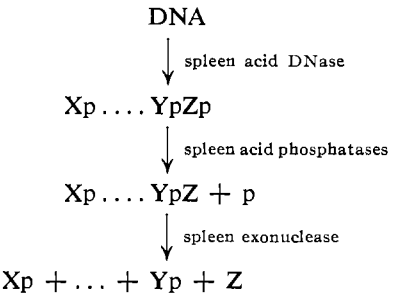

FIGURE 1: Sequence of enzymatic digestions used in this work.

Size Determinations. These were done using: (a) DEAE-cellulose–urea chromatography for the separation of isopliths, (b) DEAE-cellulose chromatography for the separation of nucleotides and nucleosides after exonuclease digestion, (c) total and monoesterified phosphorus determinations, (d) paper chromatography, and (e) Bio-Gel P-2 chromatography.

DEAE-cellulose columns were used to analyze the acid DNase digests. These were treated as described above; the final redissolution was done in distilled water (four to ten volumes were used if the original solvent was sodium acetate) and the pH was adjusted to 8.0. The columns were prepared and used according to Tomlinson and Tener (1963). DEAE-cellulose was purchased from Serva, Heidelberg, Germany; it had an exchange capacity of 0.63 mequiv/g. In most cases, linear molarity gradients of NaCl (0–0.3 M) in 0.01 M Tris-HCl buffer–7 M urea (pH 7.6) were used. The material which was not eluted by the gradient was desorbed by a higher (0.5 M) NaCl molarity step or a second NaCl (0.3–1.0 M) molarity gradient. The pH of the column effluent was 7.8–8.2; molarity gradients were checked by conductivity measurements. The flow of eluent was kept constant by using a Technicon (Chauncey, N. Y.) peristaltic pump. The ultraviolet transmission of the effluent was continuously monitored. The optical densities at 271 mμ of the fraction were measured in a Zeiss PMQ II spectrophotometer. They were corrected for the base line, which was determined by a blank run of the eluent through the column; the base line was found to increase linearly from 0.02 to 0.04 during the NaCl molarity increase from 0 to 0.3 M. The recovery of the loaded optical density was better then 95% if the base line was subtracted from the eluted optical density. In order to estimate the relative amounts of the fractions, the optical densities of the fractions which comprised the total material between successive minima were added together. This procedure obviously leads to a rather conspicuous uncertainty in the evaluation of the last fractions, owing to their poor resolution. The isolation and desalting of the fractions was carried out according to Rushizki and Sober (1962).

DEAE-cellulose columns were also used to separate nucleotides and nucleosides deriving from exonuclease digests. These were diluted to a ionic strength equal to 0.01 M and adjusted to pH 8.6; they were then loaded on columns equilibrated with 0.01 M ammonium carbonate (pH 8.6) and the nucleosides were washed through with

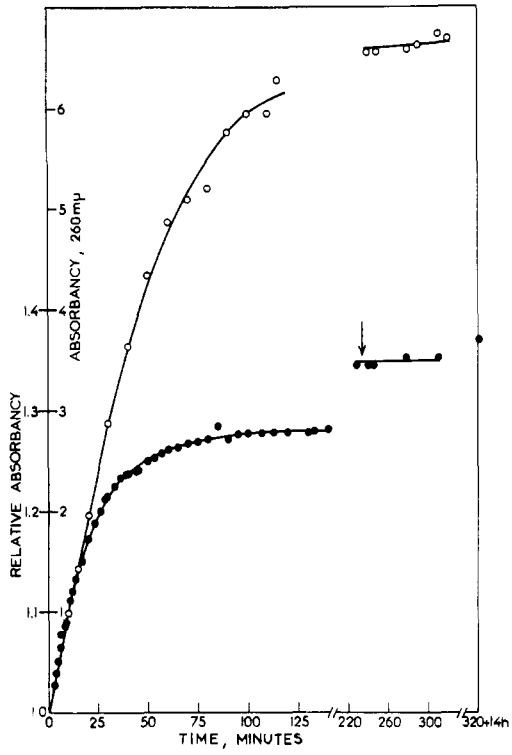

FIGURE 2: Acid-soluble oligonucleotide liberation (circles, inner ordinate; values corrected for dilution with perchloric acid) and hyperchromicity (points, outer ordinate) obtained upon digestion of calf thymus DNA with acid DNase. Silica cells with a 0.1-cm optical path were used in the experiment on hyperchromicity in order to work with the same DNA solution (A_{26c} 8.13) used in the acid-soluble oligonucleotide determination. After 225-min digestion (arrow) a second amount of enzyme was added. This figure refers to digests D and Da (see Tables I and II).

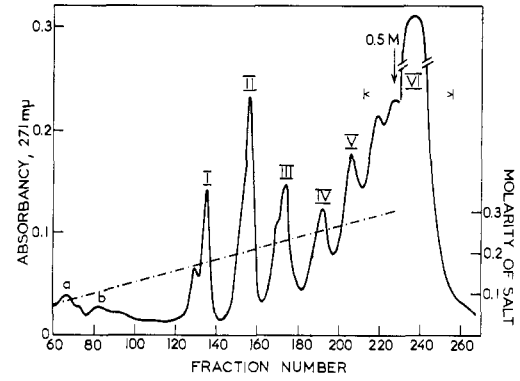

FIGURE 3: Chromatography of an acid DNase digest (A, see Tables I and II) of calf thymus DNA (170A_{271} units) on a DEAE-cellulose (chloride) column (1.8 × 25.5 cm). DNA solution (650 ml) (A_{260} 8.88) in SAB was digested with 20 μl of DNase HS 11 (undiluted, A_{280} 1.14) for 1 hr at room temperature; the sample was further digested with 10 μl of enzyme for 15 hr at room temperature. The digest was diluted with four volumes of water, adjusted to pH 8.0, and applied to the column. Collection of fractions was started immediately. Elution was carried out with a linear molarity gradient of NaCl: 0–0.3 M (2000 ml) in 7 M urea (pH 7.5). Fraction size, 8.5 ml; flow rate, 50 ml/hr. The continuous line indicates the absorption at 271 mμ; the broken line the molarity gradient.

the same buffer; nucleotides were eluted with 0.7 M ammonium carbonate (pH 8.6).

Phosphorus Determination. The method of Martin and Doty (1949) was used in the following way.

TOTAL PHOSPHORUS. Digest (0.1 ml) was incubated with 0.2 ml of 3.6 N perchloric acid for 2 hr at 200°; 0.5 ml of distilled water, 0.1 ml of 10% ammonium molybdate, and 1.3 ml of isobutyl alcohol–benzene (1:1) were added in succession to the sample. The mixture was agitated for 20 sec on a Whirlimixer (Scientific Industries, England) and the phases were allowed to separate. The top layer was pipetted and added to 0.5 ml of 3.2% sulfuric acid in absolute ethanol and 0.1 ml of 20% $SnCl_2 \cdot 1H_2O$ in concentrated HCl, freshly diluted to 200 volumes with 0.5 M sulfuric acid. The optical density at 730 mμ of the blue color which developed was measured immediately; the color was stable for at least 3 hr; 0.5 μg of phosphorus in the final 1.6-ml volume gave a reading of 0.200. Suitable blanks and standard phosphorus solutions were run simultaneously. The results obtained using this procedure were checked and found to be identical with those obtained with the methods of Chen *et al.* (1956) or of Ames and Dubin (1960).

MONOESTERIFIED PHOSPHORUS. This determination was carried out on the phosphomonoesterase digest as above, except that the initial volume of digest was 0.5 ml and no digestion with perchloric acid was done. Perchloric acid (0.2 ml of 3.6 N) was added *after* the addition of ammonium molybdate to the organic solvent mixture, and mixing was done *immediately* afterwards. The procedure permits a good extraction of phosphoric acid even in the analysis of large oligonucleotides, which are precipitated by the high concentration of perchloric acid used. Suitable blanks and standard phosphorus solutions added to nondephosphorylated digests were run in parallel.

Paper chromatography of the terminal exonuclease digest was carried out using the two-dimensional procedure of Felix *et al.* (1960).

Bio-Gel P-2 columns (Bio-Rad, Richmond, Calif.) were used to study the final exonuclease digest; since the column allows the nucleotides to be separated from the nucleosides and furthermore the latter are partially separated among themselves, this system is ideally suited for this investigation (Carrara and Bernardi, 1968).

Nucleoside Analyses. These were done by paper chromatography or Bio-Gel P-2 chromatography. These two techniques were used either to separate nucleosides and nucleotides (see above), in which case a partial (Bio-Gel P-2) or complete (paper chromatography) separation of the nucleosides was obtained, or to separate nucleosides already separated from nucleotides by DEAE-cellulose chromatography or Bio-Gel P-2 chromatography.

In order to separate isolated nucleosides we used either one-dimensional paper chromatography (second dimension of Felix *et al.*, 1960) or Bio-Gel chromatography. This is a new technique we have recently developed following the observations of Uziel and Cohn

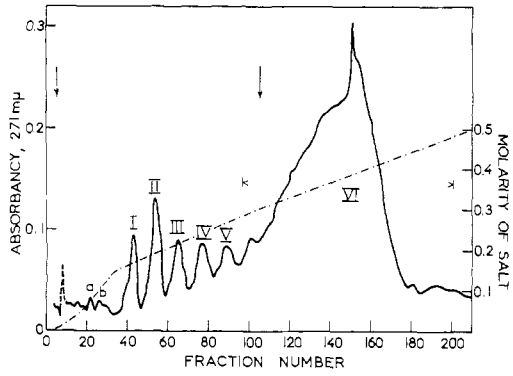

FIGURE 4: Chromatography of an acid DNase digest of chicken erythrocytes DNA (110A_{271} units) on a DEAE-cellulose (chloride) column (1.6 × 14 cm). DNA solution (30 ml) (A_{260} 14.74) in SAB was digested with 0.5 ml of acid DNase HS 10 (A_{280} 1.7; 1:160 dilution with 0.05% cytochrome *c* in SAB) for 1 hr at 37°; the sample was further digested with 0.3 ml of enzyme for 1 hr at 37°. The digest was diluted with four volumes of water, adjusted to pH 8.0, and applied to the column. Elution was carried out with two molarity gradients of NaCl, 0–0.3 (1000 ml) and 0.3–0.5 M (1000 ml), in 7 M urea (pH 7.5). Fraction size, 8.3 ml; flow rate, 68 ml/hr. Other indications as in Figure 3. See also Tables I and II.

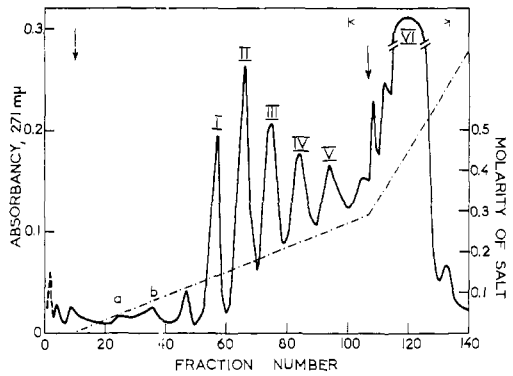

FIGURE 5: Chromatography of an acid DNase digest of *E. coli* DNA I (250A_{271} units) on a DEAE-cellulose (chloride) column (2.2 × 15 cm). DNA solution (42 ml) (A_{260} 6.1) in 0.15 M sodium acetate (pH 5.5) was digested with 0.5 ml of acid DNase HS 10 (1:100 dilution with 0.05% cytochrome *c* in SAB) for 2 hr at 37°. The digest was diluted with four volumes of water adjusted to pH 8.0 and applied to the column. Elution was carried out with two linear gradients of NaCl molarity, 0–0.3 (1800 ml) and 0.3–1 M (1200 ml), in 7 M urea (pH 7.5). Fraction size, 16.6 ml; flow rate, 68 ml/hr. Other indications as in Figure 3. See also Tables I and II.

(1965) and Schwartz *et al.* (1965); it will be described in detail elsewhere (Carrara and Bernardi, 1968).

Results

Acid DNase digestions were followed by measuring the absorption increase at 260 mμ (hyperchromic shift) and the release of acid-soluble oligonucleotides. Results obtained in a typical experiment are shown in Figure 2. Both hyperchromic shift and acid-soluble oligonucleotide formation show a biphasic curve, characterized by an initial steep slope followed by a much shallower one. The ratio of the two slopes is of the order of 100:1 in both cases; however, the acid-soluble oligonucleotide release reaches the slow phase later than the hyperchromic shift, a finding explained by the disruption of the secondary structure of fragments in acid medium. The slow phase is not due to inhibition of the enzyme by the reaction products nor to enzyme inactivation, since independent experiments have ruled out these two possibilities. When digestions were done using very small amounts of enzyme, a lag time was evident in both hyperchromic shift and acid-soluble oligonucleotide release.

These results, which confirm similar ones by Bernardi and Sadron (1964a,b) and Bernardi and Griffé (1964), indicate that three phases can be distinguished in the acid DNase digestion as followed by hyperchromic shift and/or acid-soluble oligonucleotide formation: (a) a lag phase, during which there is a precipitous molecular weight decrease, but no hyperchromic shift or oligonucleotide release; this is the initial part of the degradation already investigated from the kinetic point of view by Bernardi and Sadron (1961, 1964a,b); (b) a fast phase, with a rapid absorption increase and oligonucleotide release; and (c) a slow phase, where these two phenomena continue at a much slower rate. Table I[2] shows the hyperchromic shifts and, in some cases, the acid-soluble oligonucleotide releases caused by acid DNase, as determined on several different DNA preparations. Most of the hyperchromic shifts range from 32 to 36%, indicating that digestions were stopped at different stages of the slow phase. Slightly lower values were found for *E. coli* DNA, compared to the other DNA samples. The molar absorption at 271 mμ/oligonucleotide phosphorus present in the completely acid-soluble digests obtained during the slow phase was shown to be 7300, whereas the molar absorption at 260 mμ in perchloric acid solution was confirmed to be 9100 (Bernardi and Griffé, 1964). These digests could be dialized against running, distilled water using $^{32}/_{32}$ Visking dialysis tubings (pretreated with 0.1 M EDTA (pH 7.0) for 4 hr at 37°; G. L. Cantoni, personal communication) with an optical density recovery greater than 95%.

DEAE-cellulose Chromatography. The results obtained by fractionating several different DNA samples digested by acid DNase on DEAE-cellulose columns are presented in Figures 3–6 and in Table II.

The chromatograms obtained show the following common features: (a) an initial region of small and ill-defined peaks, which represent about 3% of the total A_{271} recovered (corrected for the base line). When loaded on DEAE-cellulose columns equilibrated with 0.01 M ammonium carbonate (pH 8.6), only about half of this material was retained. Generally, three peaks could be seen in this region of the chromatogram. A first sharp peak, indicated by a broken line in the figures, was

[2] The following nomenclature for calf thymus DNA digests has been used in the tables. (a) DNase digests are indicated by capital letters, which may be followed by small letters for re-digested samples; (b) phosphomonoesterase digests are indicated by adding numbers to the capital letters indicating the starting DNase digests.

TABLE II: Chromatography of Acid DNase Digests on DEAE-cellulose Columns.[a]

DNA Digest	Fractions (%)						
	Lower	I[b]	II	III	IV	V	VI
Calf thymus (A) (Figure 3)	4.1	3.5	6.7	5.7	5.0	7.9	67.1
Calf dephosphorylated (D) (Figure 7)	1.6	4.0	6.6	6.6	6.5	8.6	66.0
Chicken erythrocytes	3.0	2.5	4.5	2.9	3.3	4.0	79.4
E. coli I	3.0	3.0	4.9	4.7	5.2	6.5	72.7
II[c]	3.2	4.3	5.1	5.4	5.5	7.0	69.5
III	5.0	4.7	6.7	6.7	7.3	7.2	62.4
H. influenzae	3.7	3.6	7.7	7.3	8.0	8.0	61.7

[a] See Table I for further details on the digests. [b] Fractions I–V are tri- to heptanucleotides. Fraction VI is octa- and nonanucleotides plus the large unresolved fragments. [c] Total P/monoesterified P = 9.8.

formed by opalescent fractions and corresponded to the breakthrough of the urea solution; two subsequent peaks, indicated by a and b in the figures, were identified, on the basis of their elution molarity and phosphorous analysis, as mononucleotides (probably pyrimidine and purine nucleotides) in the calf thymus DNA digest. (b) A series of well-defined peaks, formed by tri- to heptanucleotides, as shown by total phosphorus/monoesterified phosphorus and total nucleotides/terminal nucleotides ratios were characteristic. These peaks are labeled I–V in the figures. The lower peaks often showed shoulders, probably due to a partial separation of pyrimidine rich from purine-rich isopliths; the latter are known to elute at higher molarities than the former (Bartos *et al.*, 1963; Rushizki *et al.*, 1964). Since the resolution of the higher oligomers is rather poor, the relative amounts given in Table II may be slightly in error. The total amount of fractions I–V is about 25–35% of the digest. These peaks are generally followed by one or two peaks (octa- and nonanucleotides) which are so poorly resolved that they have been considered, in Table II, together with the last fraction. (c) A very large, unresolved fraction, forming 65–75% of the total digest, is eluted at NaCl molarities higher than 0.3 M; generally this fraction has been eluted by a 0.5 M NaCl step (Figures 3 and 6) or by a NaCl molarity gradient of 0.3–1 (Figure 5). The average size of the oligonucleotides present in this fraction is certainly higher than 10, since it follows the octa- and nonanucleotide peaks.

Some differences were found by comparing chromatograms obtained with DNAs derived from different sources. This phenomenon, however, has not yet been studied in sufficient detail to rule out effects due to differences in the experimental conditions used.

By running dephosphorylated acid DNase digests (Figure 7) on DEAE-cellulose columns, only a slight

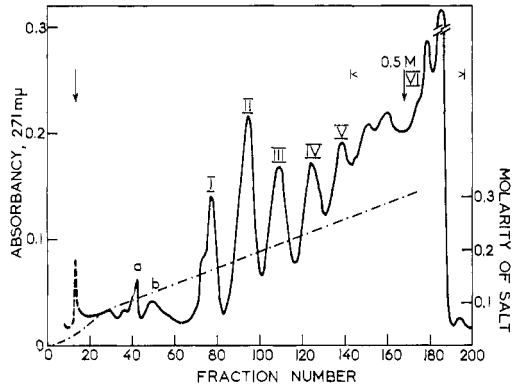

FIGURE 6: Chromatography of an acid DNase digest of *H. influenzae* DNA (111A_{271} units) on a DEAE-cellulose (chloride) column (16 × 15 cm). DNA solution (50 ml) (A_{260} 1.94) in SAB was digested with 0.5 ml of DNase HS 10 (1:100 dilution with 0.05% cytochrome *c* in SAB) for 1 hr at 37°; the sample was further digested with 0.5 ml for 3 hr at 37°. The digest was diluted with four volumes of water, adjusted to pH 8.0, and applied to the column. Elution was carried out with a linear molarity gradient of NaCl, 0–0.3 M (1000 ml), in 7 M urea (pH 7.5); 0.5 M NaCl was then used to elute the oligonucleotides left on the column. Fraction size 5.5 ml; flow rate, 68 ml/hr. Other indications as in Figure 3. See also Tables I and II.

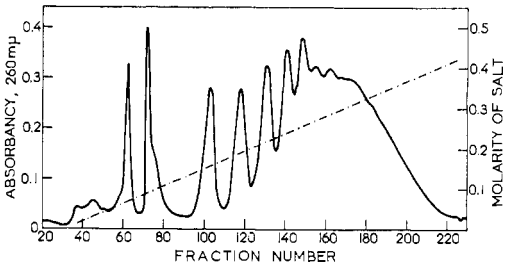

FIGURE 7: Chromatography of a dephosphorylated acid DNase digest D of calf thymus DNA (212A_{271} units) on a DEAE-cellulose (chloride) column (1.3 × 43 cm). DNA solution (300 ml) (A_{260} 8.13) in SAB was digested with 0.2 ml of concentrated acid DNase HS 17–18 (A_{280} 4.4) for 5 hr at 25°. Inactivated digest (30 ml) was further digested in the same solvent with 1 ml of phosphomonoesterase I (activity/ml = 8) for 1 hr at 37°. After inactivation of the enzyme, 25 ml of the digest diluted 1:10 with distilled water and adjusted in pH 8.0 was applied to the column. Elution was carried out with linear molarity gradient of NaCl, 0–0.5 M (2500 ml), in 7 M urea (pH 7.5). Fraction size, 8.6 ml; flow rate, 50 ml/hr. Other indications as in Figure 3. See also Tables I and II.

TABLE III: Phosphomonoesterase Digestions.[a]

Digest	Enzyme Concn (units/ml)	Solvent (M)	Digestion Time (hr)	Oligonucleotide Concn A_{271}	Oligonucleotide Size[b] Total P / Term P	Oligonucleotide Size[b] Total Nucleotides / Term Nucleotides
		A				
A1	0.004	SAB	12	9.84	11.4	
A2	0.01	SAB + NaCl (0.05)	10	9.1	10.5	
A3	0.01	SAB	18	7.0	10.5	
A4	0.01	NaAc (0.4)	19	13.3	13.2	
A5	0.0036	SAB	12	4.4	10.6	8.8
B4	0.083	NH_4Ac (0.10)	2	4.4	12.0	8.7
B5	0.17	NH_4Ac (0.075), pH 6.0	5	9.78		12.8
Ca2	0.091	SAB	14	7.75		10.8
D1	0.267	SAB	1	8.5		11.0
Da1	0.033	NH_4Ac (0.05)	5	8.84	11.2	9.4
		B				
B3	0.01	NH_4Ac (0.1)	1.5	9.78	20.4	19.5
Ca1	0.039	SAB	2.0	7.75		19.8
		C				
B1	0.01	NH_4Ac (0.05), pH 5.5	16.5	11.0	5.8[c]	
B2	0.012	NH_4Ac (0.05), pH 5.5	39	4.2	6.4	

[a] These were carried out at 37° on the acid DNase digests referred to in Table I, using acid phosphomonoesterase I, except in the case of samples Da1 and Db1, which were digested acid phosphomonoesterase II; pH was 5.0, except where otherwise stated. [b] These data were obtained after exonuclease digestion; see Table IV. [c] The ratio was equal to 12 after 2.5-hr digestion.

improvement in the resolution of the fractions was obtained, compared with the results just mentioned. The relative proportions of the fractions were the same as those obtained with nondephosphorylated digests (Table III).

Phosphomonoesterase Digestion. The average size of the oligonucleotides in the "terminal" digest obtained by acid DNase was estimated by determining the total phosphorus/terminal phosphorus ratio, using acid phosphomonoesterases I and II. The result obtained ranged from 10 to 12 (Table IIIA). Similar results were obtained by determining the total nucleotides/terminal nucleotides ratio after exonuclease digestion of the dephosphorylated acid DNase digests (Tables IIIA and IVA; see also below).

Two different sets of data are reported in Table IIIB,C. The first reports results concerning digests in which the fragment size determination gave values close to 20 instead of 10–12 as in the cases mentioned above. The larger size of these digests was due to incomplete phophomonoesterase digestion (rather than to incomplete DNase digestion), as shown by the fact that phosphomonoesterase digestion was very mild and by the comparison of samples Ca1 with Ca2 and of B3 with B4 and B5, which were obtained from the same DNase digests, Ca and B, respectively, The second set of data (Table IIIC) shows two examples of digests obtained by using long digestion times with 0.05 M ammonium acetate buffer (pH 5.5) as the solvent. They are presented here since they help to understand results described by previous authors. In these cases definitely lower fragment sizes, of the order of 6, were found. These lower values appear to be incorrect since the size, as determined after phosphomonoesterase digestion, is very much lower than the size calculated from the DEAE-cellulose chromatograms. In fact, in order to get an average size of about 6, the large fraction forming the majority of the digest, would have a size of about 7; this is clearly impossible since the large fraction follows the nonanucleotide peak. On the other hand, an average size of 10–12 for the total digest corresponds to a size of 14–15 for the large unresolved peak, which seems to be a reasonable value. The lower values seem to be due to an exonuclease contamination of the phosphomonoesterase preparation used; it is relevant to point out that nucleosides were shown to be formed during extensive phosphomonoesterase treatment of DNase digests and that exonuclease is much more active in 0.05 M ammonium acetate (pH 5.5) than in SAB.

Figure 8 shows the kinetics of dephosphorylation; the upper curve refers to a digest showing a final total P/monoesterified P equal to 10.5 (digest A2); the second

TABLE IV: Exonuclease Digestion.[a]

Digest	Solvent (M)	Digestion Time (hr)	Oligonucleotide Concn (A_{271})	Hyperchromic Shift (260 mμ) (%)	Oligonucleotide Size: Total P / Term P	Oligonucleotide Size: Total Nucleotides / Term Nucleotides	3′-P-Terminal Nucleotides (%)[b]: G	A	T	C
				A						
A5	SAB	12	4.4	25.7	10.6	8.3 (DEAE) 7.4 (P2)	42.8	33.5	17.5	6.2
A5′[c]		12	4.4	26.0		8.8 (P2) 9.8 (DEAE)	43.5	38.5	12.1	5.9
B4	NH_4Ac (0.05)	12	9.78	22.6	12.0	10.4 (P-2)	36.1	42.8	14.7	6.7
B5	NH_4Ac (0.075), pH 6.0	3.5	9.78	21.3		12.8 (DEAE)[d]	35.2	52.0	7.8	5.1
D1[e]	NH_4Ac (0.05)	3	8.5	22.5		11.05 (paper)	44.7	37.5	12.8	5.0
Da1[e]	NH_4Ac (0.05)	4	8.1	20.7	11.7	10.4 (P-2)	39.9	39.5	9.62	5.5
Ca2	SAB	3	7.75	29.0	10.8	10.8 (paper)	40.0	34.1	13.1	12.8
				B						
Ca1	SAB	3	7.75	29.7		19.8 (paper)	52.8	47.2	Trace	0.0
				C						
B4′[f]	NH_4Ac (0.05)	12	8.20	21.0		5.24 (P-2) 6.8 (paper)	28.4 26.5	31.0 40.7	40.6[g] 20.5	 12.2

[a] Digestion temperature was 37° except for sample B4 and B4′ which were digested at 25°. pH was 5.5 except for sample B5 and those in SAB. Enzyme concentrations were 0.8 and 0.65 unit/ml (A, C, D series). [b] All determinations done in duplicate. [c] Redigestion with exonuclease of sample A5. [d] Second dimension of Felix *et al.* (1960) after separation of nucleosides from nucleotides on DEAE column. [e] Sample dialized after dephosphorylation; see Figure 9. [f] Exonuclease digestion of a B4 dephosphorylated sample obtained from P-2. [g] This value refers to C + T.

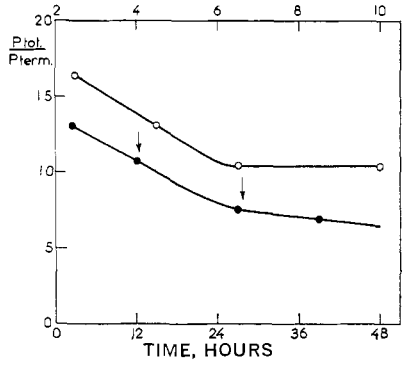

FIGURE 8: Kinetics of dephosphorylation of acid DNase digests A2 (circles and top scale) and B2 (points and bottom scale); see also Table III. Arrows on the lower curve show subsequent enzyme additions.

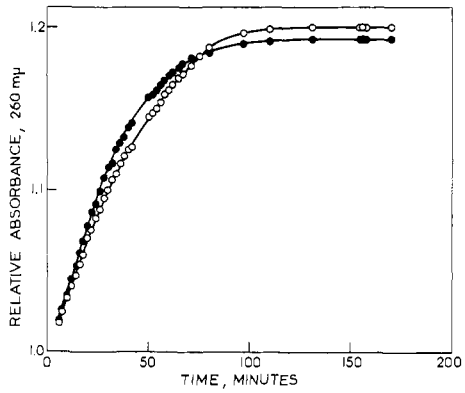

FIGURE 9: Hyperchromic shift obtained upon digestion with spleen exonuclease of dephosphorylated acid DNase digests D1 (circles) and Da1 (points); see also Table IV. Each of dephosphorylated samples (5 ml) D1 and Da1 (see Table III) was dialyzed for 4 hr at 4° against running, distilled water (this caused a loss in A_{260} of 5 and 7%, respectively), dried at 25° in a rotary evaporator, redissolved in 0.05 M ammonium acetate (pH 5.0), and digested with 50 μl each of concentrated spleen exonuclease. The hyperchromic shift was 22.5 and 20.7% for D1 and Da1, respectively.

one to a case in which a lower value (6.4) was found (digest B2).

Exonuclease Digestion. The results obtained by digesting the dephosphorylated acid DNase digests with exonuclease are shown in Table IV. The hyperchromic shifts occurring during the exonuclease digestion (Figure 9) varied, according to the different acid DNase digests, from 21 to 26%. The hyperchromic shifts were fairly constant for each acid DNase digest and they were larger when the shifts caused by acid DNase were lower. The sum of the hyperchromic shifts provoked by acid DNase and exonuclease was between 56 and 58%, at least for calf thymus DNA. The over-all hyperchromic shift shown by a DNA sample upon DNase–exonuclease digestion was close to 70%. As far as the average size of the oligonucleotides is concerned, the ratios of total nucleotides/terminal nucleotides (Table IV), as determined after phosphomonoesterase and exonuclease digestion, were systematically slightly lower than those derived from phosphorus analysis. The difference between the two sets of determinations seems to be due to a small extent of dephosphorylation occurring during the concentration of the samples prior to the nucleoside–nucleotide analysis (see below). The phosphomonoesterase contamination of exonuclease, which also might explain this result, was ruled out by experiments in which acid DNase digests were degraded with exonuclease with no formation of nucleosides; for instance, samples A5 and A5′ of Table IVA show that redigestion with exonuclease does not cause a decrease in the total nucleotides/terminal nucleotides ratio.

The 3′-phosphate-terminal nucleotides were determined in the exonuclease digests just described. Digests showing an average oligonucleotide size of 10–12 exhibited a predominance of A and G; T and, particularly, C were present at much lower levels. As shown in Table IVA, a considerable scatter in the relative proportions of the terminal nucleotides was found. The average values were in the following ranges: G 40–44%, A 34–42%, T 10–15%, and C 5–7%. Results obtained by paper chromatography appeared to be less reliable than those obtained by Bio-Gel P-2 chromatography. The scatter of the analytical results is certainly not due to the methods used since the reproducibility of duplicate analyses was satisfactory. It must therefore arise from real difference in the digests. The main sources of these differences seem to be: (a) the fact that acid DNase digests were obtained at different stages of the slow phase; (b) exonuclease contamination of phosphomonoesterase; and (c) lability of both mono- and oligonucleotides. As far as this last point is concerned, both dephosphorylation of 3′-phosphate–deoxyribonucleotides and degradation of 3′-phosphate–oligonucleotides were shown to take place during the concentration procedures. These phenomena occurred to a very small extent when using our procedures, but were serious when concentration was done from the 2 M ammonium carbonate or 2 M ammonium formate buffers used by previous authors. Detailed results on the lability of mono- and oligonucleotides carrying a 3′-phosphate group will be published elsewhere (M. Carrara and G. Bernardi, in preparation). Table IVB shows that in the case of incomplete dephosphorylation with apparent large average size only A and G were found as terminal nucleotides; this seems to be due to the fact that, at least in our hands, it was impossible to detect by paper chromatography the very small amounts of C and T, rather than to the absence of these terminals.

Finally Table IVC shows the results obtained with the digests showing a low average size (see above). Clearly, in this case a much larger percentage of both C and T is found in the terminal position. As already mentioned, Tables I–IV report detailed data obtained under different experimental conditions concerning the digests studied in the present work in order to permit precise cross-references between tables, text, and figures.

Discussion

Acid DNase Digestion. As far as acid DNase digestion is concerned, both hyperchromic shift and acid-soluble

TABLE V: 3′-Phosphate-Terminal Nucleotides.

Nucleotide	Koerner and Sinsheimer (1957) *a*	Vanecko and Laskowski (1961) *b*	Doskocil and Sorm (1962) *c*	Vanecko and Laskowski (1962)					Present Work
				c	*a*	*a*	*c*	*a*	*c*
G	33.3	23.3	36.3	68	53	43	31	34	36–43
A	30.3	38.4	27.3	15	16	28	32	35	34–43
T	28.8	26.9	24.7	9	18	17	25	21	10–15
C	7.6	11.4	11.7	8	13	12	12	10	5–7
Av size	10	4.5	4	95	54	17	6.2		10–12

[a] After venom exonuclease digestion. [b] After pancreatic DNase digestion. [c] After phosphomonoesterase and spleen exonuclease digestion. Data of Doskocil and Sorm (1962) were calculated from results given by those authors.

oligonucleotide formation are biphasic, a finding strongly reminiscent of results reported by Koerner and Sinsheimer (1957), who found that the initial rapid release of monoesterified phosphate was followed by a much slower hydrolysis for which no end point could be observed. We agree with Koerner and Sinsheimer (1957) that the slow phenomenon is due to acid DNase, since the presence of even a trace level of contaminating exonuclease (which might, alternatively, explain the results) is ruled out by the fact that no increase in the small amount of mononucleotides was evident when digestion times were longer or enzyme concentrations were higher (see Figures 3–7 and Tables I and II).

It seems that acid DNase can split very slowly, but still in an endonucleolytic way, some linkages of the fragments present in the digest. It was already mentioned that the slow phase is not due to inhibition of the enzyme by the reaction products nor to enzyme inactivation. The slow degradation brings about a shift to the left in the DEAE-cellulose chromatogram, without a significant increase of the fractions preceding the trinucleotide peak.

The degradation of large fragments into smaller ones caused by further DNase action during the slow digestion phase explains the results shown in Figure 9. Samples D1 and Da1, differing only in that the latter was more extensively digested by acid DNase than the former (see Table I and Figure 2), were dephosphorylated and digested with exonuclease. It can be seen from Figure 9 that the initial digestion rate is faster for sample Da1, which had smaller average size and, therefore, a larger number of free ends. If the additional degradation was caused by an exonuclease contaminant in acid DNase, the initial rate for sample Da1 would have been slower. The hyperchromicity shown by sample Da1 was smaller than that of sample D1, a result which is expected because the former had already undergone a more extensive degradation by acid DNase.

Fragment Size. The results shown in Figure 2 and Table I indicate that the acid DNase digests which were investigated in this work were at different stages of the slow digestion phase. As already mentioned the average oligonucleotide size was found to range from 10 to 12 for different digests. It is important to point out that this value was determined on the *total digests*, without eliminating the fragments smaller than trinucleotides. This value is close to that (10) found by Koerner and Sinsheimer (1957) at the end of the fast digestion phase. Other authors have reported much smaller average sizes of the "terminal" digest; Doskocil and Sorm (1961b) found a size of about 4; Vanecko and Laskowski (1961) a size of 4.5; Vanecko and Laskowski (1962) of 6.2; these latter values were determined, however, after elimination of mono- and dinucleotides from the digest. The difference between our results and theirs is very important and is particularly striking if the large fragments are considered. For instance, Doskocil and Sorm (1961b) report that their highest fraction (average size 7) formed only 4% of the digest, whereas in our case the large fragments (having a size higher than 7) formed 65–75% of the digest. Other discrepancies with the results of previous authors concern the mono- and dinucleotide fractions. These were found to form 7.3 and 3.3% (Laurila and Laskowski, 1957), 13.2 and about 5% (Doskocil and Sorm, 1961b, 1962), and 3 and 2% (Vanecko and Laskowski, 1962) of the digest, respectively; in contrast, in our case, all the material, preceding the trinucleotide peak in the DEAE-cellulose chromatograms was close to 3%.

The lower average size of the digests investigated by previous workers is due, in our opinion, to one or more of the following reasons. (a) Contamination of phosphomonoesterase by exonuclease; we have shown that this may be checked by comparing the average size of the DNase digest as determined after phosphomonoesterase digestion with the size as estimated from the DEAE-cellulose chromatograms; (b) contamination of exonuclease with phosphomonoesterase, reported, for instance, by Doskocil and Sorm (1961b); (c) contamination of acid DNase by spleen exonuclease; (d) dephosphorylation of 3′-phosphate–deoxyribonucleotides during the concentration step preceeding the analysis of the nucleoside–nucleotide mixture; (e) breakdown of 3′-P–deoxyribooligonucleotides during the concentration

procedures. The latter two phenomena seem to have escaped the attention of previous authors.

3'-Terminal Nucleotides. Since our DNase digests were obtained at different stages of the slow degradation phase, it is not surprising that some scatter was found in the 3'-phosphate-terminal nucleotides. Obviously, the degradative phenomena during the concentration procedures and the phosphomonoesterase digestion which we could not avoid completely, and a certain level of analytical error also contributed to the scatter. In spite of all these facts, it is quite clear from our results that deoxyadenylic and deoxyguanylic acid predominate at the 3'-phosphate positions; they are present in about equal amounts and form about 80% of the terminal nucleotides. This result explains the strong inhibition exerted by actinomycin on the degradation of native DNA by acid DNase (G. Bernardi, unpublished results).

The 3'-terminal nucleotides found by previous workers and ourselves are shown in Table V. It is unfortunate that we have no idea of the scatter of the results obtained by other authors since only single results were reported. It is evident, however, that the most relevant difference between our data and the previous ones is that we find that pyrimidine nucleotides form, at most, 20% of the 3'-phosphate termini, whereas a value almost twice as high was reported by previous investigators. This result is not surprising in view of the various artifacts incurred by them, since it corresponds to the expected randomization of the termini. Interestingly enough, we have obtained similar results in the "low-size" digests, in which degradations other than by acid DNase took place (Table IVC). At this point, one may wonder whether the amounts of C and T found in the digests of sizes 10–12 would not have been lower if we could have avoided altogether the parasytic phenomena mentioned above, and if acid DNase digests stopped just at the end of the fast phase had been used. This problem is currently being investigated in our laboratory.

In conclusion, acid DNase appears to be, in contrast with the picture presented by earlier investigations, an enzyme endowed with a narrow specificity, as shown by the large size of its "terminal" digest and by the quality of the 3'-terminal nucleotides. Final conclusions on the specificity of acid DNase must await the results concerning the 5'-OH-terminal nucleotides.

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

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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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