Glucosylated Nucleotide Sequences from T2-Bacteriophage Deoxyribonucleic Acid

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Phages T2, T4 and T6 contain glucose attached as α -glucosyl, β -glucosyl and α -gentiobiosyl groups to the hydroxymethylcytosine of the DNA (Lehman & Pratt, 1960; Kuno & Lehman, 1962). Although each type of phage DNA contains virtually the same amount of hydroxymethylcytosine, there are large differences in the total amounts of glucose and in the amounts of each of the three types of glucosylated hydroxymethylcytosine.

In T2-phage DNA, 24–28 % of the total hydroxymethylcytosine is not glucosylated; 5–6 % is substituted with α -gentiobiosyl residues and the remainder bears α -glucosyl groups (Lehman & Pratt, 1960). It has been shown (Kornberg, Zimmerman & Kornberg, 1961) that the hydroxymethylcytosine nucleotides are glucosylated after they have been incorporated into DNA, but it is not known whether the glucose is randomly distributed among these nucleotides or whether its attachment to a hydroxymethylcytosine residue is affected by the nature of the neighbouring nucleotides. We have investigated these possibilities by studying the nucleotide products obtained by digesting T2-phage DNA with diphenylamine in aqueous formic acid (Burton & Petersen, 1960) and with pancreatic deoxyribonuclease. A preliminary note describing part of this work has been published (Lunt & Burton, 1962).

MATERIALS AND METHODS

Bacteriophage. Two wild-type strains of T2 phage were used: T2(K) phage was from Dr D. Kay, Department of Pathology, Oxford, and T2(H) phage originated from Dr Hershey and was obtained via Dr G. L. Brown, King's College, London. The phages were prepared by lysis of *Escherichia coli* strain B grown in 1 l. batches of medium [composition (per l.): KH_2PO_4 , 2.1 g.; Na_2HPO_4 , 12.5 g.; NH_4Cl , 2.0 g.; MgSO_4 , 0.8 g.; and glucose, 1.8 g., added after separate autoclaving] in a rotating-flask apparatus of the type described by Mitchell (1949). The bacteria were grown to a cell density of 2×10^8 /ml. and sufficient phages were added to give a multiplicity of infection of 2–3. After

a further 5 hr. aeration the cultures were kept at room temperature for 12–36 hr. The phages were purified by differential centrifuging (Kay, 1959).

Bacteriophage deoxyribonucleic acid. The DNA was prepared according to the method of Mandell & Hershey (1960) and was dialysed against distilled water before chemical degradation, or against 0.01M-NaCl before enzymic digestion.

Enzymes. Prostatic phosphomonoesterase was prepared by methods based on those of Boman (1955) and Privat de Garilhe, Cunningham, Laurila & Laskowski (1957), as described by Burton & Petersen (1960). *E. coli* phosphomonoesterase was obtained as chromatographically purified material suspended in 65 % saturated $(\text{NH}_4)_2\text{SO}_4$ solution (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). The suspension was diluted with 2 vol. of 0.01M-MgCl₂, dialysed against 0.01M-tris buffer, pH 8.0, containing MgCl₂ (0.01M), heated at 85–90° for 10 min. and quickly chilled. Spleen phosphodiesterase was prepared by a modification (Razzell & Khorana, 1961) of the method of Hilmoe (1960); a sample was kindly given by Mr M. G. Smith. Snake-venom phosphodiesterase was prepared by Burton & Petersen (1960). Twice-crystallized pancreatic deoxyribonuclease and glucose oxidase ('Type II purified' material) were obtained from Sigma Chemical Co., St Louis 18, Mo., U.S.A. Peroxidase was from Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Phosphate analyses. The method of Chen, Toribara & Warner (1956) and a smaller-scale adaption of this method (Burton & Petersen, 1960) were used.

Purine-bound deoxyribose. This was measured by the method of Burton (1956).

Glucose estimations. Nucleotides containing hydroxymethylcytosine were rechromatographed on Whatman no. 1 paper with propan-2-ol-water (1:1 or 7:3, v/v); the ultraviolet-absorbing areas were cut out and the nucleotides removed by downward elution with water. Corresponding blank areas of paper were similarly treated. After checking the ultraviolet absorption and total phosphate content of the eluates, appropriate samples were placed in hard-glass tubes (7.5 cm. $\times$ 1 cm. internal diam.) and conc. HCl was added to give a final concentration of 1N. The tubes were then heated at 100° for 1–2 hr. to hydrolyse the glucosidic linkages. Water and HCl were removed by evaporating to dryness in a stream of air and then leaving the tubes *in vacuo* over solid NaOH overnight. To each tube was added 0.1 ml. of water and 0.3 ml. of the glucose oxidase-peroxidase reagent of Huggett & Nixon (1957). The mixtures, together with suitable standards, were incubated at 37° for 30 min. and the extinctions at 420 m μ measured. This procedure is suitable for 1–10 μ g. of glucose.

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If sufficient material was available (as with T2-phage DNA or diphenylamine digests) it was not necessary to evaporate the acid hydrolysates to dryness. Instead they were neutralized with NaHCO_3 solution and the ratios glucose: total phosphate measured on suitable samples.

Diphenylamine digests. T2-phage DNA was degraded with 2% diphenylamine in 67% (v/v) formic acid; the amine and formic acid were removed by ether extraction, and the purines by treatment with Dowex 50 (X8; H^+ form) (Burton & Petersen, 1960). For paper chromatography, the mixtures were treated with phosphomonoesterase by using the prostatic enzyme as described by Burton & Petersen (1960) or, where indicated, the *E. coli* phosphatase. For the latter enzyme, the digest solution was concentrated to about 2 ml. and MgCl_2 was added to give a final concentration of 0.01M. The digest was treated with successive amounts of enzyme until no more inorganic phosphate was liberated. After treatment with either enzyme, the mixtures were deproteinized by shaking with chloroform and centrifuging.

Ion-exchange resins. Dowex 1 and Dowex 50 resins were prepared as described by Burton & Petersen (1960). DEAE-Sephadex A-25 (medium) was washed according to the maker's instructions and converted into the HCO_3^- form by treatment with 1M- NaHCO_3 . It was washed with water and stored at 4° until used.

Paper electrophoresis. The apparatus and formate buffer, pH 2.7, were as described by Petersen (1963).

Desalting of nucleotide solutions. Ammonium hydrogen carbonate was removed from solutions by sublimation in a rotary evaporator. Ammonium formate was removed by sublimation in a freeze-drying apparatus. Solutions of

nucleotides from chromatogram papers containing ammonium sulphate were desalted on Celite-charcoal columns (Petersen, 1963).

Identification and estimation of nucleotides. Millimolar extinction coefficients for thymidylic acid and thymidine (pH 1-2) were taken to be 9.0 at 260 $\text{m}\mu$ and 6.3 at 280 $\text{m}\mu$ (Burton & Petersen, 1960). For hydroxymethyldeoxycytidylate the value at 284 $\text{m}\mu$ and pH 1-2 was taken as 12.5 (Kuno & Lehman, 1962). Based on this value and our own measurements we have used the values of 4.7 at 260 $\text{m}\mu$ and 12.0 at 280 $\text{m}\mu$. At pH 7.5 we have used the values of 6.09 at 260 $\text{m}\mu$ and 7.5 at 280 $\text{m}\mu$, these being based on the values given by Jesaitis (1957). For other deoxynucleotides the extinction coefficients are from Burton (1959). To assist identification of the products we have calculated expected extinction ratios (E_{280}/E_{260}) from the extinction coefficients of the expected or known components without allowing for any possible effects due to hypochromicity or the presence of glucosyl residues. The isosbestic point of thymidylic acid and hydroxymethyldeoxycytidylic acid (pH 1-2) was taken to be 271 $\text{m}\mu$, and 9.4 was used as the millimolar extinction coefficients at this wavelength. Where appropriate, the total pyrimidine content was determined from the extinction at 271 $\text{m}\mu$.

RESULTS

Components of diphenylamine digests

Stability of glucosidic linkages to diphenylamine-formic acid reagent. Glucose was determined both before and after hydrolysis with N-hydrochloric

Table 1. *Pyrimidine derivatives obtained from T2-phage deoxyribonucleic acid by diphenylamine degradation and treatment with phosphomonoesterase*

Compounds are numbered as in Fig. 1 except for compound 13 which was obtained (see the text) after chromatography on Dowex 1 (formate form). The calculated E_{280}/E_{260} ratios are those expected from the component nucleosides without allowing for any hypochromic effects. The digest of T2(K)-phage DNA was treated with prostatic phosphomonoesterase and that of T2(H)-phage DNA with *E. coli* phosphomonoesterase. Evidence for the assignment of sequences is given in the text and in Tables 2 and 3.

Compound no.	E_{280}/E_{260} ratio		P/Py ratio		Glc/H ratio	Moles of pyrimidine/100 g.atoms of DNA P		Identity
	Found	Calc.	Found	Calc.		Phage T2(K)	Phage T2(H)	
1	0.73	0.70	—	—	—	7.15	7.90	T
2	0.67	0.70	—	—	—	4.53	4.64	T ₂ P
3	0.67	0.70	—	—	—	2.30	2.16	T ₃ P ₂
4	0.68	0.70	—	—	—	0.82	0.98	T ₄ P ₃
5	—	0.70	—	—	—	0.49	0.41	T ₅ P ₄
6A	2.43	2.55	0	0	0	1.17	1.38	H
6	2.61	2.55	0	0	1.04	2.52	2.67	GlcH
7A	1.28	1.34	0.45	0.50	0	0.40	0.49	TpH
7	1.40	1.34	0.52	0.50	1.05	2.42	2.34	Tp(GlcH)
8A	—	—	—	—	—	0.11	0.11	?HpT
8	1.28	1.34	0.51	0.50	0.91	2.31	2.34	(GlcH)pT
9	1.03	1.08	0.69	0.67	1.02	2.19	2.26	(GlcH)T ₂ P ₂
9A	—	—	—	—	—	—	0.22	?HT ₂ P ₂
10	0.98	0.98	0.74	0.75	Present	—	2.35	(GlcH)T ₃ P ₃
10A	—	—	—	—	—	—	0.12	?HT ₂ P ₃
11	2.50	2.55	0.51	0.50	0.50	—	2.75	(GlcH)pH
12	1.69	1.65	0.72	0.67	Present	—	2.21	H ₂ Tp ₂ (Glc)
13	2.50	2.55	0.66	0.67	0.32	—	0.2*	(GlcH)pHpH

* Approximate value.

acid in the original DNA and in diphenylamine digests. No hydrolysis of the glucosidic linkages was detected during the treatment with diphenylamine and formic acid.

Paper chromatography of pyrimidine nucleotide products. Diphenylamine digests were treated with phosphomonoesterase and the pyrimidine nucleotides were separated by two-dimensional chromatography as described and illustrated in Fig. 1. The amounts of each product were calculated from the extinction coefficient in 0.03N-hydrochloric acid at 271 m μ , and the E_{280}/E_{260} ratio was used to determine the proportions of the two pyrimidines (Table 1). The various components were also analysed for phosphate and glucose.

Despite their very different histories, the two strains of T2 phage gave closely similar results, indicating that the pattern of glucosylation does not result from a contaminating phage that could conceivably have been adventitiously introduced into one of the phage stocks.

The use of different phosphomonoesterases for the digests from the two strains was not expected to influence the results and the chromatograms gave no evidence for incomplete removal of phosphomonoester groups. Any appreciable diesterase activity in either type of enzyme preparation would have been clearly shown by the experiments of Hall & Sinsheimer (1963) or Burton & Petersen (1960) (cf. also Burton, Lunt, Petersen & Siebke, 1963). From our experience we prefer

the *E. coli* phosphatase because it appeared that complete dephosphorylation was obtained more readily than with the prostatic enzyme.

Hydroxymethylcytosine nucleotides from diphenylamine digests. Nucleotides containing hydroxymethylcytosine as the only base were isolated from diphenylamine digests by chromatography on Dowex 1 (formate form) (Fig. 2). Formic acid (0.9N) eluted two nucleotide peaks (I and II; Fig. 2) and these contained hydroxymethylcytosine as the only base. The fractions from each peak were pooled and the formic acid and water removed on a rotary evaporator. After treatment with prostatic phosphomonoesterase to remove terminal phosphate groups, the two peaks were subjected to two-dimensional paper chromatography with the solvents described in Fig. 1.

Hydroxymethyldeoxycytidine was the only compound detected in the material from peak II. Peak I contained three ultraviolet-absorbing components with compositions GlcH, H₂pGlc and H₃p₂Glc respectively.* Elution of Dowex 1 (formate form) with 0.9N-formic acid thus resolves the nucleotides containing hydroxymethylcytosine as the only base into two groups according to whether the components are glucosylated or not. This system does not separate by chain length, since material from herring DNA gives one composite peak of cytosine nucleotides (Burton *et al.* 1963).

The structure of the glucosylated dinucleotide H₂pGlc (corresponding to compound 11 in Table 1) was further investigated by hydrolysis with purified spleen phosphodiesterase, an enzyme which releases nucleoside 3'-phosphates. The results in Tables 2 and 3 indicate that the dinucleotide consists of only one sequential isomer of structure (GlcH)pH.

The glucosylated trinucleotide H₃p₂Glc (compound 13 in Table 1) was hydrolysed by snake-venom phosphodiesterase, which releases 5'-nucleotides, the products being glucosylated nucleoside and unsubstituted mononucleotide in the proportions 1:2.1 (Table 2). Traces of hydroxymethyldeoxycytidine were also found, but these probably arose from the 5'-mononucleotide through the action of 5'-nucleotidase, which was a slight

* The following abbreviations are used, the prefix 'd' being omitted since all nucleosides and their derivatives are of the deoxyribose series: A, deoxyadenosine; G, deoxyguanosine; H, 5-hydroxymethyldeoxycytidine; T, thymidine; Py, pyrimidine deoxyriboside; Pu, purine deoxyriboside; Glc, glucosyl residue; GlcH, 5-(α -glucosidomethyl)-deoxycytidine; p, phosphate residue. When p is placed on the left of a nucleoside symbol it represents a 5'-phosphate and when on the right a 3'-phosphate. Compounds whose sequences are unknown or which may consist of a mixture of sequential isomers are designated by empirical formulae of the type H₃p₂Glc, HTp etc.

Fig. 1. Two-dimensional paper chromatogram of the products obtained from T2-phage DNA by degradation with diphenylamine in formic acid and treatment with phosphomonoesterase. The solvents were: first dimension, propan-2-ol-water (7:3, v/v); second dimension, 2-methylpropan-2-ol-conc. HCl-water (11:1:3, by vol.). The papers were developed as described by Burton & Petersen (1960). The analytical data for the compounds containing hydroxymethylcytosine are given in Table 1.

contaminant in the diesterase preparation. No glucosylated mononucleotide was detected. It was concluded that the trinucleotide consists of only one sequential isomer of structure (GlcH)pHpH. In agreement with this conclusion, treatment of the trinucleotide with spleen phosphodiesterase yielded glucosylated mononucleotide, unsubstituted mononucleotide and nucleoside in approximately equimolar amounts (Table 2).

Dinucleotides containing thymine and hydroxymethylcytosine from diphenylamine digests. Paper chromatography indicated the presence of four of these dinucleotides in diphenylamine digests. For further study fractions IV and V obtained by chromatography on Dowex 1 (formate form) (Fig. 2) were desalted and treated with phosphomonoesterase. Two-dimensional paper chromatography showed that fraction IV yielded compounds

7 and 8 (Table 1), whereas peak V yielded compound 7A. Hydrolysis of compounds 7A and 8 with spleen phosphodiesterase (Table 2) indicated that they were TpH and (GlcH)pT respectively.

Compound 7 was very resistant to hydrolysis by spleen phosphodiesterase and gave only small amounts of thymidylic acid and GlcH; in one experiment only 4.6% was hydrolysed, whereas, under the same conditions, 76% of compound 8 and 83% of compound 11 were hydrolysed. Compounds 7 and 8 had the same composition and their relative chromatographic positions were analogous to those of the two isomers thymidylyl-(3'-5')-deoxycytidine and deoxycytidylyl-(3'-5')-thymidine (cf. Burton & Petersen, 1960). Compound 7 is therefore assigned the structure Tp(GlcH).

The unglucosylated nucleotide HpT has not been identified with certainty but traces of ultraviolet-

Table 2. *Amounts of products formed by the action of phosphodiesterases on oligonucleotides from diphenylamine digests of T2-phage deoxyribonucleic acid*

The compounds (see Fig. 1 and Table 1) were incubated at 37° with 20 μ moles of sodium succinate buffer, pH 5.8, 1 drop of toluene and spleen phosphodiesterase (5–10 units; Hilme, 1960) in a total volume of 0.6 ml. for 2–48 hr. Compound 13 was treated with snake-venom phosphodiesterase under the conditions used by Burton & Petersen (1960). The products were separated on paper chromatograms with the solvents described in Fig. 1 and the amounts recovered are shown. Percentage hydrolysis was calculated from the proportion of unhydrolysed starting material recovered. (See Table 3 for analytical data on the principal products.)

Substrate			Products						Identity of substrate
Compound no.	Composition	Percentage hydrolysed	T (μmole)	Tp (μmole)	H (μmoles)	Hp (μmole)	GlcH (μmole)	(GlcH)p (μmoles)	
Spleen phosphodiesterase									
13	H ₃ p ₂ Glc	95	—	—	0.57	0.54	0.06*	0.42	(GlcH)pHpH
11	H ₂ pGlc	90	—	—	2.19	—	—	2.04	(GlcH)pH
		100	—	—	0.36	—	0.09*	0.23	
8	HTpGlc	100	0.22	—	—	—	—	0.19	(GlcH)pT
7	HTpGlc	4.6	—	0.045	—	—	0.042	—	Tp(GlcH)
7A	HTp	100	0.011*	0.013	0.021	—	—	—	TpH
Snake-venom phosphodiesterase									
13	H ₃ p ₂ Glc	98	—	—	0.05*	0.68†	0.32	—	(GlcH)pHpH

* In these experiments the nucleoside is believed to have been formed through hydrolysis of the corresponding mononucleotide.

† Assumed to be the 5'-phosphate.

Table 3. *Analytical data for products containing hydroxymethylcytosine*

For details see Table 2. The amount of hydroxymethylcytosine was determined from the extinction at 271 m μ .

Diesterase substrate (Table 2)	Analyses on product			Identity
	E_{280}/E_{260} ratio	P/H ratio	Glc/H ratio	
	Spleen phosphodiesterase			
8	2.64	1.01	0.79	(GlcH)p
11	2.50	1.10	0.88	(GlcH)p
11	2.59	0	0	GlcH
13	2.55	0	0	H
13	2.56	1.00	0.60	(GlcH)p
13	1.99	0.97	0	Hp
	Snake-venom phosphodiesterase			
13	2.57	0	0.90	GlcH
13	2.81	0.98	0	pH

absorbing material (compound 8A) were found on the paper chromatograms in the expected position relative to that of compound 7A, i.e. TpH.

Frequency of purine-purine sequences. The frequency of these sequences is deduced to be 25.3% since this was the proportion of total phosphate released as inorganic phosphate by the action of diphenylamine on T2(H)-phage DNA. The value is close to that of 26.3% which is indicated by the results of Josse, Kaiser & Korn-

berg (1961), who determined nearest-neighbour frequencies with the aid of DNA polymerase.

When diphenylamine digests were subsequently treated with the *E. coli* phosphatase, the inorganic phosphate rose to 73.7% of the total. The residual organic phosphate at this stage represents the interpyrimidine phosphate and it is in satisfactory agreement with the interpurine phosphate.

Components of pancreatic deoxyribonuclease digests

Degradation of T2-phage deoxyribonucleic acid by pancreatic deoxyribonuclease. The conditions chosen were those under which Mg^{2+} and Ca^{2+} ions activate the enzyme synergistically (Wiberg, 1958; Stone & Burton, 1962). A solution (100 ml.) containing 0.5 mg. of pancreatic deoxyribonuclease, 50 mg. of T2-phage DNA, sodium chloride (10 mM), magnesium chloride (6 mM), calcium chloride (6 mM) and toluene as preservative was incubated at 37° for 72 hr. During this time three further 0.5 mg. amounts of enzyme were added and the pH was maintained at 7.5 by the addition of 0.01N-sodium hydroxide from an automatic titrating apparatus (Radiometer, Copenhagen, Denmark). The ratio of inorganic phosphate released by phosphomonoesterase to the purine-bound deoxyribose indicated that about 38% of the 3'-phosphoester bonds had been hydrolysed by the deoxyribonuclease.

Isolation and identification of nucleotides. The components of the digest were separated on a column of DEAE-Sephadex (Pharmacia, Uppsala) as described in Fig. 3. After being desalted, the material in each of peaks 2, 3 and 4 was fractionated two-dimensionally on Whatman 3MM paper as described in Fig. 4.

All dinucleotides except G_2P_2 were isolated. TG_2P_2 was contaminated by another component (possibly HT_2P_2) and was purified by paper chromatography in ethanol-1M-ammonium acetate (6:25, v/v). The separation procedures did not resolve isomeric dinucleotides nor did they separate glucosylated dinucleotides from corresponding non-glucosylated compounds. The nucleotides were characterized by their ultraviolet-absorption spectra at pH 7.5 and phosphate content, and by the proportion of the total phosphate that could be released by prostatic phosphomonoesterase (Table 4). The total dinucleotides isolated accounted for 31% of the purine and pyrimidine bases in the digest.

Dinucleotides of a purine and hydroxymethylcytosine. These components were further identified by base analysis (Hershey, Dixon & Chase, 1953), and from their contents of purine-bound deoxyribose and glucose (Table 4). The hydroxymethylcytosine in these two fractions is only partly glucosylated and, since the products of pancreatic-

Fig. 2. Separation of pyrimidine nucleotides from diphenylamine-degraded T2-phage DNA on Dowex 1 (formate form). After removal of purines by treatment with Dowex 50 (H^+ form), the digest (174 mg. of DNA) was made alkaline with ammonia, adsorbed to a column (20 cm. $\times$ 0.9 cm. diam.) of Dowex 1 (formate form) and washed with water. Peaks I and II were removed by elution with 0.9N-formic acid. The remaining compounds were eluted with the linear gradient obtained by using 1 l. of 2N-formic acid in the mixing vessel and 1 l. of 4N-formic acid containing ammonium formate (0.4M) in the reservoir; 5 ml. fractions were collected.

Fig. 3. Separation of nucleotides obtained by the action of pancreatic deoxyribonuclease on T2-phage DNA. The digest from 50 mg. of T2(K)-phage DNA was adjusted to pH 8.6 and adsorbed on to a column (40 cm. $\times$ 0.9 cm. diam.) of DEAE-Sephadex (HCO_3^- form). The column was washed with water and the elution pattern shown was obtained with a linear gradient of 0.0-0.3M-ammonium hydrogen carbonate, pH 8.6, in 3 l.

Table 4. Analytical data for dinucleotides isolated from pancreatic deoxyribonuclease digests of T2-phage deoxyribonucleic acid

The numbers of the compounds correspond to those in Fig. 4. The amounts are based on the extinction at 260 mμ and pH 7.5. The extinction coefficients were calculated as the sum of the extinction coefficients for the corresponding mononucleotides; no correction was made for hypochromicity. The amounts given are the mean values obtained from two different enzymic digests. The E_{260}/P values were obtained in a separate experiment.

Compound no. (Fig. 4)	Source on DEAE-Sephadex column	E_{260}/E_{260} ratio		E_{260}/E_{260} ratio		$E_{260}/\text{total P}$ ratio		Glc/H ratio	Identity	Moles isolated/100 g. atoms of DNA P
		Found	Calc.	Found	Calc.	Found	Calc.			
1	Peak 2	0.82	0.80	0.17	0.14	14.2	15.3	—	A_2P_2	1.86
2	Peak 2	0.81	0.82	0.47	0.45	10.4	10.7	0.56	AHP_2^*	2.42
3	Peak 2	0.94	0.90	1.22	1.23	6.3	6.09	0.48	H_2P_2	0.42
4	Peak 3	0.98	0.95	0.40	0.38	13.5	14.05	—	AGP_2^*	1.28
5	Peaks 3 and 4	1.05	1.05	0.85	0.84	9.30	9.45	0.57	GHP_2^*	1.00
7	Peak 2	0.75	0.75	0.94	0.92	7.8	7.6	—	HTP_2	1.63
8	Peak 2	0.74	0.75	0.36	0.35	12.0	12.2	—	ATP_2	2.80
9	Peak 4	0.96	0.93	0.65	0.68	10.8	10.95	—	GTP_2	2.10
10	Peak 2	0.65	0.65	0.68	0.70	8.5	9.10	—	T_2P_2	2.03
									Total	15.54

* Both glucosylated and unglucosylated forms are present (see the text and Table 6).

deoxyribonuclease action are 5'-phosphates (Volkin, Khym & Cohn, 1951), the following components could be present in each purine-containing dinucleotide fraction: p-Pu-p-(GlcH), p-Pu-p-H, p-(GlcH)-p-Pu and p-H-p-Pu respectively. To investigate their components, each dinucleotide fraction was degraded with 2% diphenylamine in 67% formic acid. After removal of the amine and formic acid by ether extraction the products were separated on paper and analysed as shown in Tables 5 and 6. Diphenylamine treatment of each dinucleotide fraction gave the expected purine and each of the four possible hydroxymethyldeoxycytidine phosphates. In addition, the dinucleotide fraction containing guanine gave a trace of material

Fig. 4. Fractionation of dinucleotides from pancreatic deoxyribonuclease digests. The material from each of peaks 2, 3 and 4 from DEAE-Sephadex columns (Fig. 3) was desalted and applied to Whatman 3MM paper. The components were separated by electrophoresis in formate buffer, pH 2.7 (Petersen, 1963), in one dimension, followed by chromatography in a second dimension with saturated ammonium sulphate-propan-2-ol-water (40:1:9, by vol.) for 28 hr. Several unidentified compounds from peak 4 were poorly resolved and their positions are not shown. The identities of the dinucleotides are given in Table 4. Compound 6 was deoxyguanosine monophosphate.

Table 5. Separation of hydroxymethylcytosine derivatives obtained from dinucleotides

Paper electrophoresis was carried out in formate buffer, pH 2.7 (Petersen, 1963), followed by paper chromatography in 2-methylpropan-2-ol-conc. HCl-water (11:1:3, by vol.) in the second dimension. Electrophoresis was at 420 v for 17 hr.

Product	Electrophoretic mobility	
	(cm./hr.)	R_F
pHp	1.5	0.57
p(GlcH)p	1.3	0.39
pH	0	0.53
p(GlcH)	0	0.32

Table 6. *Diphenylamine degradation of dinucleotides containing a purine and hydroxymethylcytosine*

After treatment with diphenylamine and formic acid, total P was measured on the digests. The remainder (6.0 μ moles of total P from AHP₂ or 2.2 μ moles from GHP₂) was extracted with ether and applied to Whatman no. 1 paper for separation as shown in Table 5. The amounts of products are expressed as moles recovered/mole of dinucleotide, each value being the mean of results obtained for material from two different enzymic digests. The deviations from the mean are indicated for the amounts of hydroxymethylcytosine compounds recovered. The total amounts of these compounds recovered were 86% for the adenine dinucleotide and 84% for the guanine dinucleotide.

Product	Amount	Organic P/H ratio	Glc/H ratio	Structure of parent dinucleotide
From adenine dinucleotides				
pHp	0.311 $\pm$ 0.003	1.86	0	pHpA
p(GlcH)p	0.430 $\pm$ 0.007	1.93	0.87	p(GlcH)pA
pH	0.021 $\pm$ 0.004	0.97	0	pApH
p(GlcH)	0.076 $\pm$ 0.025	0.95	0.85	pAp(GlcH)
P _i	0.135*	—	—	pApH + pAp(GlcH)
From guanine dinucleotides				
pHp	0.275 $\pm$ 0.011	2.02	0	pHpG
p(GlcH)p	0.436 $\pm$ 0.027	1.93	0.88	p(GlcH)pG
pH	0.079 $\pm$ 0.013	1.04	0	pGpH
p(GlcH)	0.067 $\pm$ 0.008	1.03	0.83	pGp(GlcH)
P _i	0.19*	—	—	pGpH + pGp(GlcH)

* P_i was determined separately before paper chromatography in only one of the experiments.

containing equal amounts of hydroxymethylcytosine and phosphate. This material was found in the area of the chromatogram in which gentiobiosylhydroxymethyldeoxycytidine 5'-phosphate would be expected to occur.

With digests of both dinucleotide fractions, about 85% of the total hydroxymethylcytosine applied to the papers was recovered in the four mononucleotides. In agreement with Sinsheimer (1955), dinucleotides with sequence p-Py-p-Pu predominate strikingly over those of the reverse sequence. Only the nucleotides of the reverse sequence liberate inorganic phosphate in the diphenylamine degradation. Direct estimation of the inorganic phosphate in the mixture of products therefore provides an accurate measure of the ratio of the two types of sequence in each dinucleotide fraction since it is not affected by losses or possible hydrolysis during the subsequent manipulations and chromatography. The phosphate analyses described in Table 6 indicate that 86–87% of the hydroxymethylcytosine-adenine dinucleotides and 81% of the hydroxymethylcytosine-guanine dinucleotides have the sequence p-H-p-Pu. In all four sequences hydroxymethylcytosine was present in both the glucosylated and unglucosylated forms but the nature of the purine did not significantly affect the degree of glucosylation in the p-H-p-Pu dinucleotides. There was not enough material to draw any firm conclusion for the dinucleotides p-Pu-p-H.

Dinucleotides of a purine and thymine. These were also examined by diphenylamine degradation. The ratio of thymidylic acid to thymidine 3',5'-diphosphate in the products indicated that 62% of

the adenine-thymine dinucleotide and 65% of the guanine-thymine dinucleotide had the sequence p-T-p-Pu.

Hydroxymethyldeoxycytidylic dinucleotide. The fraction H₂p₂ was treated with phosphomonoesterase and chromatographed on paper in 2-methylpropan-2-ol-conc. hydrochloric acid-water (11:1:3, by vol.). Only one nucleotide component was detected and the glucose:hydroxymethylcytosine ratio was found to be 0.48. This is consistent with the compound being identical with the (GlcH)pH obtained after degradation with diphenylamine. Our failure to obtain the completely unglucosylated product could be due to the rarity of the HpH sequence or possibly to its hydrolysis by the deoxyribonuclease.

DISCUSSION

The results show that glucosyl residues are not randomly distributed among the hydroxymethylcytosine residues of T2-phage DNA. Analysis of the products obtained by diphenylamine degradation (Table 1) showed that, despite their very different histories, two strains of T2 phage exhibited essentially the same pattern of glucosylation. Glucosylation is restricted in sequences of two or three adjacent hydroxymethylcytosine nucleotides which are terminated at both ends by purine nucleotides. Unglucosylated residues also occur in sequences of composition H, HTp, HT₂p₂ and HT₃p₃, and which, in the original DNA, were terminated at both ends by purine nucleotides. The amounts obtained (Table 7) indicate that the presence of a purine nucleotide on both

Table 7. *Effects of neighbouring purines on glucosylation*

Sequence in DNA	Compounds derived from sequence (Table 1)	GlcH/H* ratio in sequence
Pu-p-H-p-Pu	6, 6A	0.67
Pu-p-H-p-T-p-Pu	8, 8A	0.96
Pu-p-T-p-H-p-Pu	7, 7A	0.85

* Average values, obtained from the results in Table 1.

sides of a hydroxymethylcytosine nucleotide hinders glucosylation much more than a purine nucleotide at only one side. The amounts of glucose in the sequences

Pu-p-H-p-T-p-Pu and Pu-p-T-p-H-p-Pu

suggest that a purine nucleotide linked through the 5'-position of hydroxymethyldeoxycytidine restricts glucosylation less than one linked through the 3'-position.

To decide whether adenine and guanine nucleotides have different effects when linked to the 3'-position of a hydroxymethyldeoxycytidine residue, the nucleotides released by the action of pancreatic deoxyribonuclease on T2-phage DNA were examined. Other work on the products obtained with pancreatic deoxyribonuclease has been reviewed by Becking & Hurst (1963). In our experiments, 38% of the 3'-phosphoester bonds were hydrolysed by the action of comparatively large amounts of enzyme in the presence of two activating metal ions (Ca^{2+} and Mg^{2+} ions; Wiberg, 1958). The extent of hydrolysis, the amounts of the resulting dinucleotides and the proportions of the various isomeric dinucleotides generally resemble those reported by Becking & Hurst (1963) for the corresponding nucleotides released from calf-thymus DNA by pancreatic deoxyribonuclease activated by Mn^{2+} ions. Much smaller amounts of dinucleotides have been obtained when the enzyme was activated by Mg^{2+} ions without the addition of Ca^{2+} ions (e.g. Sinsheimer, 1954).

The dinucleotides obtained by enzymic hydrolysis of T2-phage DNA amounted to about 31% of the total DNA phosphorus and hence 15.5% of the total possible dinucleotide sequences in the DNA. In agreement with Sinsheimer (1955) we have found more of the p-Py-p-Pu type of dinucleotide than of each of the other three types, namely p-Pu-p-Py, p-Pu-p-Pu and p-Py-p-Py. From the amounts of P_i reported in Table 6 and the results of Josse *et al.* (1961), we can conclude that the dinucleotide products contain 34% of the HpA and 27% of the HpG sequences known to be present in T2-phage DNA. The analyses do not indicate any appreciable difference in the extent to which these sequences are glucosylated.

A more striking restriction of glucosylation was found in tracts of adjacent hydroxymethylcytosine nucleotides terminated at both ends by purine nucleotides. Each tract has only one glucosyl residue and this is attached to the first of the hydroxymethylcytosine residues when the sequences are written in the conventional notation. We have not detected corresponding nucleotides containing more or less glucose so that such sequences can only be present in small amounts.

Lehman & Pratt (1960) found that 24–28% of the hydroxymethylcytosine in T2-phage DNA is not glucosylated. Taking the total content of this pyrimidine to be 17 moles/100 nucleotides (Wyatt & Cohen, 1953; Hershey *et al.* 1953), this amounts to 4.1–4.76 moles of unglucosylated hydroxymethylcytosine/100 nucleotides. The amounts of products isolated and characterized from diphenylamine digests (Table 1, excluding compound 12) account for 3.2–3.3 moles of these unglucosylated hydroxymethylcytosine residues.

We did not fully characterize any product that contained thymine together with more than one hydroxymethylcytosine nucleotide. Sequences of adjacent hydroxymethylcytosine nucleotides in these components may conceivably possess the same pattern of glucosylation as do those that are terminated at both ends by purine nucleotides. Each tract of adjacent hydroxymethylcytosine nucleotides in the DNA would then contain only one glucose residue and there would be one unglucosylated hydroxymethylcytosine for every nearest-neighbour pair of hydroxymethylcytosine nucleotides, e.g. $(\text{GlcH})-(\text{pH})_n$ contains n unglucosylated hydroxymethylcytidine residues and n nearest-neighbour pairs. According to Josse *et al.* (1961), there are 3.4 such nearest-neighbour pairs/100 deoxynucleotides, and so they would contain 3.4 unglucosylated hydroxymethylcytosine residues. Together with the unglucosylated hydroxymethylcytosine residues found in other types of sequences (1.7/100 deoxynucleotides; Table 1), they would amount to 5.1 unglucosylated hydroxymethylcytosine residues. Considering the various experimental errors, this value is not inconsistent with the results of Lehman & Pratt (1960).

α -Gentiobiosylhydroxymethylcytosine residues (Lehman & Pratt, 1960) have not been identified among the products isolated from diphenylamine digests, suggesting that such residues occur predominantly in the longer sequences that have not yet been resolved by our chromatographic procedures.

It has been reported that the unglucosylated hydroxymethylcytosine residues in T2-phage DNA cannot be glucosylated even *in vitro* by the α -glucosyltransferase that is induced on infection by

phage T2 (Kornberg, Zimmerman, Kornberg & Josse, 1959; Kornberg *et al.* 1961; Pratt, Kuno & Lehman, 1963). Our results show that the glucose residues are not distributed randomly in T2-phage DNA and suggest that the action of the glucosylating enzyme at different sites is strongly influenced by the nature of the bases of the neighbouring nucleotides. In some instances (e.g. see Table 7), neighbours on both sides of the hydroxymethylcytosine nucleotide appear to have appreciable effects. It also appears that the enzyme may act only very slowly on hydroxymethylcytosine nucleotides that are attached through their 5'-positions to other hydroxymethylcytosine nucleotides. This possibility is consistent with observations reported by Josse & Kornberg (1962) on the glucosylation of a synthetic polymer consisting of repeating hydroxymethylcytosine deoxynucleotides and repeating guanine deoxynucleotides. Up to 23% of the hydroxymethylcytosine nucleotides could be glucosylated, but the rate of glucosylation was slow, being initially only 3% of the rate occurring with a DNA which contained hydroxymethylcytosine but no glucose.

The function of the glucose in the DNA of T-even bacteriophages is not known. The glucose does not appear to be involved in the genetic code because genetic markers can be transferred from one phage to another one with a different glucose content (Streisinger & Weigle, 1956; Sinsheimer, 1956). Cohen (1957) suggested that the glucose may protect the phage DNA from breakdown by host enzymes in the early stages of infection, and it has since been shown that T6 phage with little or no glucose in its DNA shows very limited growth in *E. coli* strain B, although it can form plaques with relatively high efficiency on suitable strains of *E. coli* K12 (Shedlovsky & Brenner, 1963; Hattman & Fukasawa, 1963). Shedlovsky & Brenner (1963) (cf. also Symonds, Stacey, Glover, Schell & Silver, 1963) point out that these differences might arise through degradation of the unglucosylated DNA by enzymes present in *E. coli* B but not in *E. coli* K12 strains. If glucose can protect the DNA of the infecting phage from enzymic breakdown it may be significant that in T2-phage DNA the unglucosylated hydroxymethylcytosines are confined to relatively few types of nucleotide sequence.

SUMMARY

1. The frequencies of various pyrimidine nucleotide sequences in the DNA of T2 phage have been measured. The α -glucosyl residues are not distributed at random among the hydroxymethylcytosine nucleotides.

2. About 35% of the total unglucosylated hydroxymethylcytosine was found in sequences of

two or three adjacent hydroxymethylcytosine nucleotides that in the original DNA were linked to purine nucleotides at both ends of the sequences. These sequences contained only one glucosyl residue and this was attached to the first hydroxymethylcytosine nucleotide when the sequences are written in the conventional notation, i.e. with the 5'-phosphate group before and the 3'-phosphate after each nucleoside residue.

3. Single hydroxymethylcytosine nucleotides between two purine nucleotides accounted for about 23% of the total hydroxymethylcytosine and 29% of the total unglucosylated hydroxymethylcytosine nucleotides. A further 5% of the unglucosylated hydroxymethylcytosine was found in the deoxynucleotide sequences (3'-5')-purine-thymine-hydroxymethylcytosine-purine. The reverse sequences contained only about 1.2% of the unglucosylated hydroxymethylcytosine. These findings suggest that neighbouring purine nucleotides hinder glucosylation especially when linked to the 3'-position of hydroxymethyldeoxycytidine.

4. T2-phage DNA was digested with pancreatic deoxyribonuclease activated by Ca^{2+} and Mg^{2+} ions. The dinucleotides that were isolated amounted to 31% of the original DNA, and the pyrimidine-(3'-5')-purine dinucleotides predominated. The nature of the purine did not significantly affect the extent of glucosylation in the hydroxymethylcytosine-(3'-5')-purine dinucleotides.

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The Transfer of [³H]Stearic Acid from Chylomicra to Milk Fat in the Goat

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It has been thought probable for a long time that the ruminant udder uses plasma lipid as a source of milk fat in addition to synthesizing some from acetate. An arteriovenous difference of neutral fat during lactation has been reported by Lintzel (1934) in the goat and by Graham, Jones & Kay (1936) in the cow. Work with isotopes also has suggested that the ruminant udder can incorporate blood triglycerides into milk fat. Glascock, Duncombe & Reinius (1956) showed that dietary [³H]stearic acid was transferred to milk fat, as did Riis, Luick & Kleiber (1960) for colloidal plasma lipid. There is, therefore, evidence that the mammary glands take up long-chain fatty acids from the blood triglycerides. It is not, however, decisive, because of the metabolism of other organs in the whole animal and the failure to demonstrate (Glascock, McWeeny & Smith, 1957) a conventional 'precursor-product' relationship (Zilversmit, Entenman & Fishler, 1943). The attempts of Glascock (1958) to obtain more quantitative information by the intravenous injection of

labelled fat emulsions were unsatisfactory because they were rapidly removed from the circulation. Further, Glascock *et al.* (1957) reported that a goat died after an intravenous injection of gelatin-stabilized fat emulsion, and in a cow 'acute symptoms' developed after the injection of only 300 mg. of emulsion stabilized with lecithin and glycerol monostearate. Similarly, T. Gillman & W. M. F. Leat (personal communication) observed that a commercial preparation (Infonutrol) used successfully in man can cause collapse and sometimes death in adult sheep. In isolated perfused goat mammary glands we have observed a fall in blood flow and haemolysis with neutral emulsions of triglycerides and long-chain fatty acids, stabilized with bilirubin or lecithin in albumin-Krebs solution (D. C. Hardwick & J. L. Linzell, unpublished work). For this reason we do not normally include fat in our substrate mixture, although in some experiments a fall in milk fat concentration after some hours of perfusion suggests that the tissue may need it.

The aim of the present experiments was first to test the use of goat chyle (intestinal lymph) rich in chylomicra as a source of fat for the perfused goat's

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