2. After injection of ^{32}P into young rats the specific activity of the free glycerylphosphorylethanolamine after short periods was always greater than that of the phosphatidylethanolamine. This suggests that glycerylphosphorylethanolamine is not an *in vivo* breakdown product of phosphatidylethanolamine, although other considerations indicate that it does not lie on the main synthetic pathway.

3. The specific activity of a combined form of glycerylphosphorylethanolamine labile to 10% trichloroacetic acid at 4° was always identical with that of phosphatidylethanolamine. The specific activity of the ethanolamine-containing acetal-phospholipid was also similar.

4. The synthesis of radioactive glycerylphosphorylethanolamine by minced rat brain has been accomplished. No *in vitro* synthesis of phosphatidylethanolamine could be demonstrated.

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REFERENCES

Anchel, M. & Waelsch, H. (1944). *J. biol. Chem.* **152**, 501.
Ansell, G. B. & Dawson, R. M. C. (1951). *Biochem. J.* **50**, 241.
Ansell, G. B. & Norman, J. M. (1953). *Biochem. J.* **54**, viii.
Awapara, J. (1948). *Arch. Biochem.* **19**, 172.
Bakay, L. (1951). *Arch. Neurol. Psychiat., Chicago*, **66**, 419.
Campbell, P. N. & Work, T. S. (1952). *Biochem. J.* **50**, 449.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Crumpler, H. R. & Dent, C. E. (1949). *Nature, Lond.*, **164**, 441.
Dawson, R. M. C. (1953). *Biochem. J.* **53**, viii.
Dawson, R. M. C. & Richter, D. (1950). *Proc. Roy. Soc.* B, **137**, 252.
Dent, C. E. (1948). *Biochem. J.* **43**, 169.
Fowden, L. (1951). *Biochem. J.* **48**, 327.
Hahn, L. & Hevesy, G. (1937). *Skand. Arch. Physiol.* **77**, 148.
Jeffers, W. A. & Griffiths, J. Q. (1942). *The Rat in Laboratory Investigation*, eds. Farris, E. J. & Griffith, J. Q. London: Lippincott Co.
Kennedy, E. P. (1953). *J. biol. Chem.* **201**, 399.
Klenk, E. & Böhm, P. (1951). *Biochem. Z.* **288**, 98.
Kornberg, A. & Pricer, W. E. jun. (1952*a*). *Fed. Proc.* **11**, 242.
Kornberg, A. & Pricer, W. E. jun. (1952*b*). *J. Amer. chem. Soc.* **74**, 1617.
Lewis, P. R. (1952). *Biochem. J.* **52**, 330.
Lovern, J. A. (1952). *Biochem. J.* **51**, 464.
Norman, J. M. & Dawson, R. M. C. (1953). *Biochem. J.* **54**, 396.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Popják, G. & Muir, H. (1950). *Biochem. J.* **46**, 103.
Samuels, A. J., Boyarsky, L. L., Gerard, R. W., Libet, B. & Brust, M. (1951). *Amer. J. Physiol.* **164**, 1.
Schmidt, G., Benotti, J., Hershmann, B. & Thannhauser, S. J. (1946). *J. biol. Chem.* **166**, 505.
Thannhauser, S. J., Boncoddo, N. F. & Schmidt, G. (1951). *J. biol. Chem.* **188**, 423.
Walker, D. M. (1952). *Biochem. J.* **52**, 679.
Wiggins, L. F. & Williams, J. H. (1952). *Nature, Lond.*, **170**, 279.
Woiwod, A. J. (1949*a*). *Biochem. J.* **45**, 412.
Woiwod, A. J. (1949*b*). *J. gen. Microbiol.* **3**, 312.

The Bases of the Nucleic Acids of some Bacterial and Animal Viruses: the Occurrence of 5-Hydroxymethylcytosine

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Recent studies on the multiplication of viruses have directed attention increasingly toward their nucleic acids. Hershey & Chase (1952) have shown that most, if not all, of the sulphur-containing protein of coliphage T2, which appears to be present in the outer shell of the virus, does not enter the infected cell. However, deoxyribonucleic acid (DNA), apparently organized within the virus, is in some way transferred to the host cell, and appears, therefore, to participate more intimately in the transmission of genetic properties. On infection of *Escherichia coli* with bacteriophage T2, T4 or T6, there is immediate cessation of synthesis of ribonucleic acid (RNA) and net synthesis of DNA is detectable in about 10 min. (Cohen, 1947, 1951). A similar apparent redirection of DNA synthesis during virus multiplication is characteristic of certain induced lysogenic systems, but in this case synthesis of RNA

continues unimpeded (Siminovitch & Rapkine, 1952). Much work has been directed toward tracing the origin of the structural components of phage DNA (e.g. Weed & Cohen, 1951; Putnam, 1952).

It is important, therefore, to know what chemical properties the nucleic acids of bacterial and other viruses may possess. Wide variations have been demonstrated in the composition, with respect to purine and pyrimidine bases, of the RNA's of plant viruses (Markham, 1953) and the DNA's of insect viruses (Wyatt, 1952*b*).

Concerning the DNA of coliphages T2 and T6, conflicting results have appeared. Smith & Wyatt (1951) reported the presence of substantial amounts of cytosine in phage T2; Weed & Cohen (1951) reported isolation of deoxycytidylic acid from T6; Marshak (1951), however, could find only adenine, guanine and thymine in the nucleic acid of T2, and concluded that this virus contained only these three bases. In no case was the total recovery of nitrogenous bases in terms of phosphorus recorded: in the investigation of Weed & Cohen, a total analysis was not the object, and in the other two studies it was assumed that procedures found satisfactory with DNA from other sources would give quantitative results with phage DNA also.

We have re-examined the DNA's of phages T2, T4 and T6, and found that they do contain no cytosine but instead a hitherto unrecognized pyrimidine base, now identified as 5-hydroxymethylcytosine. We report the quantitative purine and pyrimidine composition of the DNA's of these viruses and also qualitative and quantitative results on some other viruses which were examined with the object of determining the distribution of the new base. Preliminary notices from this investigation have already appeared (Wyatt & Cohen, 1952, 1953).

MATERIALS

Phages T2, T4 and T6

The six viruses which provided the basis of these studies were the r and r$^+$ strains of coliphages T2, T4 and T6. Their properties and the isolation of many of the preparations used have been described (Cohen & Arbogast, 1950*b*). Various new preparations were isolated from lysates of high titre (5×10^{11}–10^{12} virus particles/ml.) obtained by growing *Esch. coli*, strain B, to 3×10^9 bacteria/ml. and infecting these cells with an average of five virus particles each. The cells grew exponentially to this level when suspended in 350 ml. of medium at 37° in a 2 l. flask rotated in such a fashion that the medium was thinly layered on its inner surface, thereby providing maximal aeration. After infection, rotation was continued until lysis. The glucose and nitrogen content of the basal mineral medium were fortified tenfold.

In addition, two large preparations of phage T6r$^+$, totalling 35 g., were generously prepared for us by Dr J. Spizizen of the Research Division of Sharp and Dohme, Inc. Because of difficulties attending the purification of large quantities of virus, these preparations contained a certain proportion of non-virus material. They contained about 20% of phage DNA.

For isolation of DNA the phages were disrupted by urea (3·6 g./10 ml. of virus suspension: Cohen, 1947) and deproteinized in M-NaCl-urea solution by $CHCl_3$:*n*-octanol (8:1, v/v). The nucleic acids were precipitated with 4 vol. of cold ethanol and washed in 80%, 90% and absolute ethanol and ether. The fibrous solids were dried *in vacuo* over P_2O_5.

Other viruses

Phage T7. Two large preparations containing *Esch. coli* phage T7 were made available to us by Dr J. Spizizen. They contained about 4% of DNA. From one of these, nucleic acid was isolated by the urea technique. A small preparation (5 mg.) of T7 virus was also provided by Dr L. M. Kozloff of the Department of Biochemistry, University of Chicago. This contained about 10% of DNA.

Phage T5. A preparation of this coliphage, containing about 26% of DNA, was the gift of Dr G. Lark, of New York University School of Medicine. From a portion of it, DNA was isolated by the procedure described by Smith & Wyatt (1951), modified by incubating in N-NaOH at room temperature (20°) instead of 37°. NaOH was used in preference to urea in case traces of RNA might be present. We established, using DNA of phage T6, that 5-hydroxymethylcytosine withstands this treatment without loss.

Polyhedral virus. Polyhedral virus from caterpillars of *Colias philodice eurytheme* Bdvl., for which we are indebted to Dr E. A. Steinhaus of the University of California, was isolated by the method of Bergold (1947, 1953).

Vaccinia virus. This material (160 mg., containing about 2·1% of DNA) was prepared for us by Dr A. L. Brown of the Research Division of Sharp & Dohme Inc., using the method of Hoagland, Smadel & Rivers (1940). Treatment of this virus with urea failed to release nucleic acid. DNA was therefore isolated by the procedure of Smith & Wyatt (1951), incubating in N-NaOH at 20°.

Meningo-pneumonitis virus. A preparation of this virus, a member of the psittacosis group, was the gift of Dr M. M. Sigel, of the Research Department, Children's Hospital of Philadelphia. The virus was isolated by centrifugation from chick allantoic fluid, and we were supplied with 17 mg., containing about 2·5% of DNA.

Pyrimidine derivatives

5-Hydroxymethylcytosine and 5-hydroxymethyluracil were synthesized at our suggestion and kindly made available to us by Dr C. S. Miller, of the Research Division of Sharp and Dohme, Inc. The former was prepared* by reduction of ethyl cytosine-5-carboxylate with $LiAlH_4$, and the latter by the method of Litzinger & Johnson (1936).

A specimen of 5-hydroxyuracil, prepared by the method of Davidson & Baudisch (1925), was the gift of Dr G. H. Hitchings. A specimen of 4-methylcytosine, prepared by Dr Hitchings by a method analogous to that used by Hitchings, Elion, Falco & Russell (1949) for cytosine, was obtained through the courtesy of Dr A. Bendich.

* A description of this synthesis is in preparation by Dr Miller and will be submitted to the *Journal of the American Chemical Society*.

EXPERIMENTAL AND RESULTS

Evidence for the presence of an unknown component in phages T2, T4 and T6

Since hydrolysis with perchloric acid (70% at 100° for 1 hr.: Marshak & Vogel, 1951) had proved satisfactory for quantitative liberation of DNA bases from insect viruses, this method was tried first for analysis of phages T4 and T6. The bases were separated by paper chromatography using an *iso*propanol:water:HCl mixture as the solvent, and eluted and estimated as previously described (Wyatt, 1951*b*, 1952*b*). The principal products were guanine, adenine and thymine, in total amount equivalent to 75–80% of the virus P. The chromatograms also showed weak spots of a substance having an R_F value equal to that of cytosine (0·46–0·50), the yield of which (1–5 moles/100 g. atoms P, using the extinction coefficient of cytosine) varied widely from one experiment to another. On elution, this substance proved to have its ultraviolet-absorption maximum at a slightly longer wavelength than has cytosine, and on being rechromatographed with 86% (v/v) aqueous *n*-butanol as the solvent its R_F (about 0·07) was much smaller than that of cytosine. The substance was largely unchanged by heating with formic acid at 175° for 2 hr. and so was unlikely to be a nucleoside. In addition, its ultraviolet-absorption spectrum exhibited a shift in alkaline solution similar to those of cytosine and 5-methylcytosine, which the ribosides and deoxyribosides of these bases lack.

It was then found that by using formic acid (88% at 175° for 30 min.) for hydrolysis of the viruses the yield of the unknown was much increased (8–12 moles/100 g. atoms P), the total recovered bases now corresponding to 85–90% of virus P. These observations suggested the presence in phage nucleic acid of a relatively labile pyrimidine base different from any previously reported.

A small amount of the substance, obtained by evaporation of the eluate of a chromatogram spot, was deaminated by treatment with HNO_2 (35 μl. of 2M-$NaNO_2$ and 7 μl. of glacial acetic acid were added and, after standing overnight at 27°, the solution was applied directly to paper for chromatography). The product bore a similar relationship, in spectral and chromatographic properties, to uracil as the parent substance did to cytosine.

The structure of the unknown was suggested to us by study of its spectral and chromatographic properties, as already briefly reported (Wyatt & Cohen, 1952, 1953). Ultraviolet-absorption spectra were read at pH 1, pH 7–8 and pH 13 (Fig. 1, Table 1). From among a number of pyrimidine bases which we examined or whose spectra have been published, only cytosine and 5-methylcytosine exhibited shifts of absorption maximum with change of pH closely paralleling those of the unknown. This suggested that our substance had the polar substituents (2-hydroxy-6-amino-) of cytosine and was additionally substituted in the 5-position. Substitution at C-4 (e.g. 4-methylcytosine) diminishes the extent of the spectral shift in alkali. Addition of an ionizable group at C-5 (e.g. 5-hydroxyuracil) changes the spectrum radically, hence the required substituent should be non-ionizable.

The behaviour of the unknown on paper chromatograms in solvents differing as to pH and to water content, as described in our earlier communications, also suggested ionizable groupings identical with those of cytosine and further indicated that the additional substituent should be hydrophilic in nature. As 5-hydroxycytosine had been eliminated, 5-hydroxymethylcytosine seemed a probable structure.

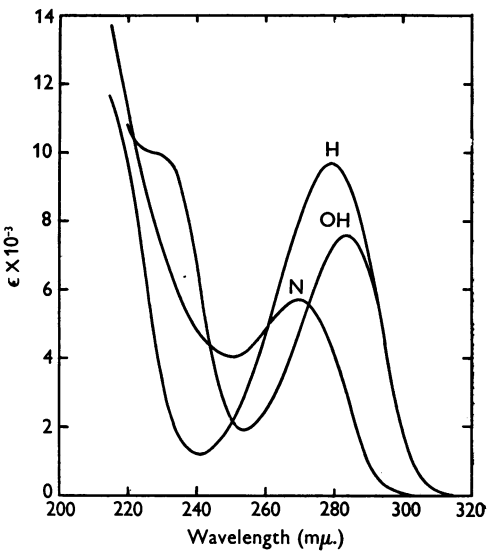


Fig. 1. Ultraviolet-absorption spectra of 5-hydroxymethylcytosine in 0·1N-HCl (H), 0·1N-NaOH (OH) and at pH 7·4 (N). ϵ is the molecular extinction coefficient. See Table 1.

Table 1. *Ultraviolet-absorption data*

(ϵ is the molecular extinction coefficient.)

		Maxima		Minima	
Substance	Solvent	Wavelength (mμ.)	ϵ	Wavelength (mμ.)	ϵ
5-Hydroxymethylcytosine	0·01M Sodium phosphate buffer (pH 7·4)	269·5	5710	251	4060
	0·1N-HCl	279·5	9700	241·5	1230
	0·1N-NaOH	283·5	7590	254	1890
5-Hydroxymethyluracil*	Buffer (pH 7·4) as above	261	—	231	—
	0·1N-HCl	261	—	231	—
	0·1N-NaOH	285	—	245	—

* Measured from eluates of chromatogram spots; the substance was not isolated in crystalline form.

Isolation and characterization of 5-hydroxymethylcytosine from phage T6

To establish with certainty the identity of the supposed new base it was desirable to isolate in pure form a sufficient quantity for elementary analysis and comparison with synthetic material. For this we were fortunate in having the large phage preparations provided by Dr Spizizen. Since the lability of the new base, unique among natural pyrimidines, was not at first fully realized, yields were much lower than they might have been. Nevertheless, a satisfactory sample was isolated by the following procedure.

From 4·5 g. of the virus concentrate, DNA was prepared by the urea technique referred to above. The product weighed 665 mg. and had N/P = 3·85 (atomic proportions).

For removal of purines (Tamm, Hodes & Chargaff, 1952), 500 mg. of T6 DNA were dissolved in 50 ml. of water and dialysed for 24 hr. at 32° against two changes (each 1000 ml.) of 0·03 N-HCl (pH 1·6), followed by dialysis for 3 days against three changes of distilled water at room temperature. The solution from inside the cellophan dialysis sac was concentrated under reduced pressure and freeze-dried. The yield of 'apurinic acid' was 235 mg., from which some 80% of the original purines had been removed without change in the ratio pyrimidines/P. It was evident, however, that some pyrimidine-containing material had passed through the dialysis membrane, and an additional yield corresponding to about 45 mg. was recovered from the last dialysis water.

220 mg. of this 'apurinic acid' were hydrolysed with 1·5 ml. of 88% formic acid at 165° for 20 min. It is clear from later experiments that the yield of hydroxymethylcytosine would have been greatly increased by use of a larger volume of formic acid. The hydrolysate was spread as a band near one edge of a sheet (18·25 × 22·5 in.) of Whatman no. 3 filter paper and chromatographed in the *iso*propanol: HCl solvent. The appropriate band was eluted and the eluate concentrated and chromatographed in a similar way with water-saturated *n*-butanol as the solvent on Whatman no. 3 paper which had been washed with distilled water. The ultraviolet absorption of the eluate from this second chromatogram indicated the presence of some 12 mg. of pyrimidine derivative.

Several fractions obtained in a similar manner, containing altogether about 30 mg. of pyrimidine derivative, were combined and treated with 60 mg. of picric acid. The picrate crystallized in needles. After removal of picric acid (by extraction with toluene and ether in the presence of H_2SO_4, the latter being then removed with $Ba(OH)_2$) the solution was concentrated and the free base crystallized in prisms (21·5 mg.). After two recrystallizations from water the product weighed 15·4 mg. Its elementary composition was compatible with the proposed structure. (Found, after drying *in vacuo* at room temperature: C, 40·2; H, 5·35. $C_5H_7O_2N_3$, 1/2 H_2O requires C, 40·0; H, 5·37%. Loss of weight on drying *in vacuo* at 100° over P_2O_5, 4·7% (required: 6·00%). Found, on exhaustively dried material: N (Dumas), 29·3. $C_5H_7O_2N_3$ requires N, 29·8%.)

At approximately the same time as the natural substance was obtained in pure form, synthetic 5-hydroxymethylcytosine was made available to us by Dr Miller. With respect to their ultraviolet-absorption spectra (Table 1, Fig. 1) and to their movement on paper chromatograms in several solvents, the natural and synthetic substances were identical. On heating, both specimens charred slowly above 200°, without melting up to 300°. 5-Hydroxymethyluracil, subsequently prepared by Dr Miller, was similarly indistinguishable from the deamination product of the natural base. We conclude that the base isolated from bacteriophage T6 is 5-hydroxymethylcytosine.

Quantitative hydrolysis of bacteriophage deoxyribonucleic acid

In view of the failure to obtain good yields of 5-hydroxymethylcytosine from DNA by hydrolysis with $HClO_4$, it was of interest to determine the effect of this acid on the pure base. Accordingly, 0·5 mg. of the synthetic base was heated with 0·05 ml. of 72% $HClO_4$ at 100° for 1 hr. Unexpectedly, 97% of the initial 5-hydroxymethylcytosine was recoverable after heating. However, since the nucleoside and nucleotide of this base are not found after hydrolysis of phage DNA with conc. $HClO_4$ at 100°, the low recovery must result from destruction and not from incomplete liberation. This loss may be due either to weakening of the pyrimidine ring by the glycosidic linkage at N-3 or to the effect of other substances in the hydrolysate, such as H_3PO_4. We have not tested these possibilities experimentally.

For quantitative analysis of the DNA of phages T2, T4 and T6, we hydrolysed with formic acid. When a proportion of formic acid to nucleic acid was used similar to that previously used for DNA from other sources, the yield of hydroxymethylcytosine was somewhat variable, and the total recovered bases were equivalent to only some 90% of the DNA P. When pure hydroxymethylcytosine was subjected to these hydrolytic conditions in the presence of thymus DNA, then separated from the other bases by 2-dimensional chromatography and estimated, only some 70–75% of the added amount was recovered (Table 2, expts. 1 and 2). Alteration of time or temperature of

Table 2. *Recovery of 5-hydroxymethylcytosine subjected to hydrolytic conditions in 88% formic acid at 175° for 30 min. in the presence of thymus deoxyribonucleic acid*

Expt. no.	Formic acid (μl./mg. DNA)	5-Hydroxymethylcytosine (moles/g. atom P) Added	Recovered	Recovery (%)
1	80	0·146	0·099	68
	80*	0·146	0·066	45
2	80	0·175	0·134	77
	80*	0·175	0·108	62
3	260	0·215	0·187	87
4	360	0·206	0·195	95
5	600	0·330	0·318	96·5

* Protein (bovine serum albumin, equal in wt. to the DNA) was also added.

hydrolysis did not lead to significant improvement, but recovery of this base was found to depend markedly on the volume of formic acid used. With a sample of 1·5 mg. of phage, or 0·7 mg. of phage DNA, for which 0·05 ml. of 88% formic acid had been used, maximal yields in terms of P are obtained with 0·25–0·5 ml. By using sufficient formic acid, recovery of hydroxymethylcytosine subjected to hydrolysis along with thymus DNA was raised to approximately 96% (Table 2), and the total base recovery from phage DNA became equivalent to 97–99% of total P.

It has also been found important to avoid an excessive volume of air above the formic acid during hydrolysis. This leads to loss of hydroxymethylcytosine, which can, however, be prevented by replacing the air in the tube with nitrogen or formic acid vapour before sealing it off. Evidently the compound is most stable under reducing conditions, and this may account for the efficacy of formic acid in liberating nucleic acid bases without loss. With sufficient formic acid in a small enough tube, as described below, however, there was no advantage in replacing the air with a non-oxidizing atmosphere.

The increased volume of formic acid was found to lead also to slightly improved yields of other bases, especially guanine, from DNA. When ox-spleen DNA was hydrolysed under these conditions total base recovery was equivalent to 98–100% of the P (Table 6). It appears that the use of an insufficient volume of formic acid has been one reason for low total recoveries of bases from DNA in some previous analyses (e.g. Wyatt, 1951*b*; Chargaff, Lipshitz, Green & Hodes, 1951; Laland, Overend & Webb, 1952).

It will be noted that the formic acid referred to above is 88% (Merck reagent), although in previous work 98–100% (A.R.) has been used. When the two grades were tested simultaneously, no significant difference was found in yields of the bases from DNA. A similar dependence of yield upon volume was found with 98% as with 88% formic acid, and the former produced a darker-coloured hydrolysate suggesting greater degradation of the deoxypentose.

The procedure ultimately adopted for base analysis of phage DNA is as follows. Virus (1·5 mg.) or virus DNA (0·7 mg.) is weighed into a Pyrex glass tube of 6 mm. internal diameter. Formic acid (0·5 ml. of 88%) is added, and the tube is sealed off about 20 mm. above the surface of the liquid and heated to 175° for 30 min. The tube is opened, the hydrolysate evaporated to dryness under reduced pressure at a temperature not exceeding about 75°, and the residue redissolved in 25 μl. of N-HCl. Two 8 μl. portions are taken for chromatography, and two 2 μl. portions for P estimation.

Elementary analyses

P was estimated in the virus and nucleic acid preparations by the method of Bergold & Pister (1948), and N by a micro-Kjeldahl procedure. For the elementary analyses on purified 5-hydroxymethylcytosine we are indebted to the analytical laboratory of the Research Division of Sharp & Dohme, Inc.

Determinations of deoxyribonucleic acid bases in viruses and other materials

Since it was possible that some of the virus preparations might contain RNA, the early chromatograms of phages hydrolysed with $HClO_4$ (which breaks down RNA as well as DNA to free bases) were examined for possible uracil. The ratio of uracil to thymine should give an approximate indication of the ratio of RNA to DNA. Maximal values for uracil, calculated from the absorption at 260 mμ. of the appropriate areas of chromatograms, ranged from 1·9 to 2·8% of the thymine for seven preparations of T4r$^+$, T4r, T6r$^+$ and T6r; in no case did the absorption curve exhibit a peak at this wavelength, so that the absorption must have been largely due to other degradation products from the virus, and any actual uracil was considerably less than the figures mentioned.

The relative proportions of the DNA bases found in the r and r$^+$ mutants of coliphages T2, T4 and T6 are shown in Table 3. The small difference in apparent thymine content of whole viruses compared with isolated DNA has no biological significance, but is due to interference by breakdown products of the non-DNA moiety of the virus. The very small differences in results from the various virus strains are not significant, but fall within the range of experimental error. Because of the lability of 5-hydroxymethylcytosine it is probable that even under the conditions of hydrolysis finally adopted a small amount of this base is lost. The results in Table 2 suggest that this may be of the order of 4%, and the figures presented may require corresponding correction.

From the ratios of total recovered bases to total P (Table 3) it is evident that in the best virus preparations the P may be fully accounted for as nucleotides. The possible presence of 1 or 2% of non-DNA P is not excluded, however,

Table 3. *Composition of phages T2, T4 and T6 with respect to DNA bases, phosphorus and nitrogen*

Material analysed	No. of preparations	No. of analyses	Mean estimated content of bases (moles/100 moles) Adenine	Thymine	Guanine	5-Hydroxymethylcytosine	Total bases/P (moles/g. atom)	N/P (atomic ratio)
T2r$^+$ DNA	2	3	32·5	32·6	18·2	16·7	0·97	—
T2r DNA	1	3	32·4	32·4	18·3	17·0	0·98	—
T6r$^+$ DNA	2	10	32·5	32·5	18·3	16·7	0·99	3·85
T2r$^+$ virus	1	4	32·0	33·3	18·0	16·8	0·99	7·0
T2r virus	1	4	32·3	33·4	17·6	16·7	0·95	7·0
T4r$^+$ virus	1	3	32·3	33·1	18·3	16·3	0·96	6·9
T4r virus	2	3	32·2	33·5	18·0	16·3	0·94	6·7
T6r$^+$ virus	3	2	32·5	33·5	17·8	16·3	0·99	7·2
T6r virus	2	3	32·3	33·4	17·7	16·6	0·88	6·7

because base estimation in the presence of protein may err slightly on the high side. In other preparations some non-DNA P is indicated: this is generally greater in the r than in the r^+ mutants, and in two preparations of T6r averaged some 10% of the total P. This is in general agreement with the results of an earlier study, which indicated that the non-DNA P is associated with contaminants of host origin (Cohen & Arbogast, 1950*a*).

As additional characterization of the viruses, N/P ratios are included in Table 3. These are very similar for the different preparations, tending, as would be expected, to be lower in those containing extraneous P. In spite of some earlier evidence that T4 may contain less DNA per infectious unit than T2 and T6 (Cohen & Arbogast, 1950*a*), no significant difference is evident in the N/P ratios of the three viruses. Our values agree well with the mean value of 6·83 calculated from the data of Herriott & Barlow (1952) for T2.

The discovery of 5-hydroxymethylcytosine in these viruses naturally raised the question whether this substance might occur elsewhere. The biological materials listed in Table 4 were examined. Formic acid hydrolysates were subjected to chromatography in two solvents, as previously described for estimation of 5-methylcytosine (Wyatt, 1951*a*), so that maximal figures for the contents of hydroxymethylcytosine relative to cytosine were obtained. In no case did the eluate of the appropriate region of the chromatogram have the characteristic absorption spectrum of hydroxymethylcytosine, so that no evidence was obtained for the presence of this substance in any of these materials. Where only a small amount of material containing a low proportion of DNA was available, the figures for maximal hydroxymethylcytosine are necessarily raised by interfering substances.

By a similar analysis of phage $T6r^+$, using 45 mg. of virus, its maximal content of cytosine was limited to 0·2% of its hydroxymethylcytosine.

During the course of this investigation, DNA preparations were obtained from phage T5 and from vaccinia virus of sufficient apparent purity to justify our reporting the molar ratios of the bases found in them. These are shown in Table 5, along with a new analysis of ox-spleen DNA. This was the preparation previously described and analysed (Wyatt, 1951*b*), which is apparently identical in composition with calf-thymus DNA, and the present analysis is considered to be more accurate because of better recoveries resulting from the use of a higher proportion of formic acid in hydrolysis. Quantitative analyses were also performed on phage T7; however, since there was evidence of interference by materials other than DNA, we do not report the figures and merely remark that the results indicated a closer approximation to equimolar proportions of the four bases than are in the DNA of phage T5.

Table 4. *Maximal contents of 5-hydroxymethylcytosine, calculated from the ultraviolet absorption of chromatogram eluates, in various biological materials*

Material examined	Wt. hydrolysed (mg.)	Maximal 5-hydroxymethylcytosine as percentage of cytosine (mol.prop.)
Dried *Esch. coli*, strain B	270	0·2
Crude DNA from *Esch. coli**	—	0·6
Ox-spleen DNA	18	0·2
Phage T5	2·1	1·0
Phage T7†	3·8	2·7
Crude DNA from phage T7‡	18	0·5
Polyhedral virus	10·6	2·4
DNA from vaccinia virus	1·2	0·6
Meningo-pneumonitis virus	14·8	3·9

* Isolated by the method of Smith & Wyatt (1951) from 400 mg. of dried bacteria.
† Preparation of Dr Kozloff.
‡ Isolated from the preparation of Dr Spizizen.

DISCUSSION

It is necessary to reconcile certain earlier results with those now presented. The report of cytosine as a component of phage T2 by Smith & Wyatt (1951) was based on a limited amount of material, and when chromatograms were obtained identical in appearance with those of DNA from other sources it was assumed without further critical examination that spots having equal R_F values represented the same substances. The mistaken recognition of deoxycytidylic acid by Weed & Cohen (1951) resulted from the following coincidence. When the products of hydrolysis of phage T6 DNA in N hydrochloric acid were separated on paper chromatograms, a band was resolved having both the R_F value and the ultraviolet-absorption maximum (278 mμ. in 0·01 N hydrochloric acid) of deoxycytidylic acid, which it was therefore assumed to be. Recent re-examination of this fraction by Dr Weed (personal communication), however, has resulted in its resolution on ion-exchange columns into two components: one with absorption maximum at 282 mμ. at pH 2, representing deoxy-5-hydroxymethylcytidylic acid, and the other, with a maxi-

Table 5. *Base composition of DNA's estimated after hydrolysis in* 400–700 *times their weight of* 88% *formic acid*

Source of DNA	No. of analyses	Moles/100 moles estimated bases: Adenine	Thymine	Guanine	Cytosine	Total bases/P (moles/g. atom)
Ox spleen	4	27·9	27·3	22·7	20·8*	1·00
Phage T5	2	30·3	30·8	19·5	19·5	0·93
Vaccinia virus	1	29·5	29·9	20·6	20·0	1·00

* 1·3 moles of 5-methylcytosine are also present (Wyatt, 1951*b*); this base was not estimated in the present analyses.

mum 274 mμ., as yet unidentified. When combined, these products had an absorption spectrum close to that of deoxycytidylic acid. The conclusions drawn from these studies, however, are not altered by the substitution of hydroxymethylcytosine for cytosine.

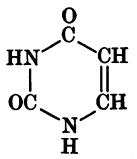
Marshak (1951) missed hydroxymethylcytosine because of his use of perchloric acid for hydrolysis along with a chromatogram solvent system in which it happens to migrate together with guanine. This accounts for the anomalous absorption spectrum for guanine which he reported.

In spite of the considerable evidence that DNA may play a specific role in the transmission of hereditary characters, we were unable to demonstrate any difference in the composition of the DNA of the r and r^+ mutants of phages T2, T4 and T6. This confirms the inference drawn from similar analyses on a number of insect viruses (Wyatt, 1952*b*) that genetic difference is not necessarily accompanied by a detectable quantitative difference in DNA composition.

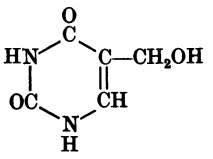
A common pattern has been noted in the composition of DNA from many sources: the molar ratios (adenine)/(thymine) and (guanine)/(cytosine + 5-methylcytosine) are relatively constant and close to unity (Chargaff, 1951; Wyatt, 1952*a*). The same regularities are seen to be valid with DNA from phage T5 and from vaccinia virus, and also with DNA of phages T2, T4 and T6 except that here cytosine is replaced by 5-hydroxymethylcytosine. Whether these near-unity ratios actually signify equal numbers of the corresponding nucleotides in the molecule is as yet uncertain. The present studies, however, have served to emphasize how quantitative errors can result from small differences in experimental conditions and purity of materials, and it is our experience that successive improvements in technique have resulted in bringing the observed ratios successively closer to unity. One is tempted to speculate that regular structural association of nucleotides of adenine with those of thymine and of guanine with those of cytosine (or its derivatives) in the DNA molecule requires that they be equal in number. There is as yet, however, no direct evidence for such a theory.*

The occurrence of 5-hydroxymethylcytosine as a major constituent of the nucleic acid of a virus, none of which could be found in the host cells, presents problems of fundamental importance for the chemistry of virus production. Although discussion must at present remain largely speculative, certain possibilities may be pointed out.

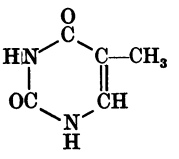
We are concerned with the following pyrimidine bases:



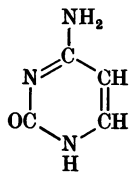
Uracil



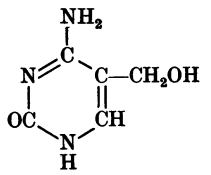
5-Hydroxymethyluracil



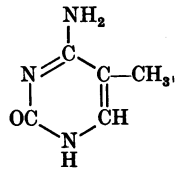
Thymine



Cytosine



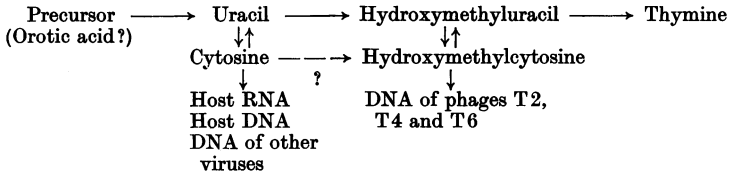
5-Hydroxymethylcytosine



5-Methylcytosine

The metabolic pathways for pyrimidines appear generally to involve their ribosides and deoxyribosides rather than the free bases, and preliminary experiments by one of us (S. S. C.) indicate that this probably is the case in *Esch. coli*. In the rat, Reichard & Estborn (1951) have demonstrated that deoxycytidine can be utilized for production of thymidine, but not vice versa. Elwyn & Sprinson (1950) have implicated the β-carbon of serine as a source of the 5-methyl group of thymine, which is evidently synthesized by methylation of a preformed pyrimidine ring. Since serine cleaves to formaldehyde, we may question whether methyl-group synthesis from serine may not involve an initial hydroxymethylation followed by reduction. If this is so, 5-hydroxymethylpyrimidines (or their deoxyribosides) could be normal metabolites, inter-

* Since this was written, a structure for DNA involving such specific pairing of nucleotides has been proposed by Watson & Crick (1953).



mediary in the synthesis of 5-methylpyrimidines, and a scheme (as above) may be tentatively proposed.

Folic acid has been implicated in the synthesis of the 5-methyl group of thymine (Goldthwait & Bendich, 1952), and it may be with the proposed hydroxymethylation step that it is concerned. This suggests a possible explanation for the inhibition of growth of phages T 2, T 4 and T 6 by sulphanilamide under conditions (including the presence of thymine) which do permit multiplication of phages T 1, T 3 and T 7 (Rutten, Winkler & de Haan, 1950). If the drug interferes with hydroxymethylation, so long as thymine is provided, only the growth of viruses requiring a hydroxymethylpyrimidine will be inhibited. It was with this in mind that we examined the virus of meningo-pneumonitis, which is also sensitive to sulphonamides. It does not contain hydroxymethylcytosine, hence its inhibition must be otherwise accounted for, and may be due to another function of folic acid. The theory with respect to phages T 2, T 4 and T 6 is neither supported nor necessarily invalidated.

The proposal that 5-hydroxymethylcytosine can be derived from cytosine is supported by recent tracer experiments with *Esch. coli* infected with phage T 6 (Weed & Cohen, unpublished). Two routes are possible, however: (*a*) the hydroxymethyl substituent may be added directly to cytosine (or its deoxyriboside), or (*b*) cytosine may be deaminated to uracil, which would be hydroxymethylated and then aminated to produce hydroxymethylcytosine. In the latter case 5-hydroxymethyluracil would be a precursor both of thymine and of hydroxymethylcytosine, and the last compound could be an abnormal end product produced only during growth of one of the viruses requiring it.

In either case the problem arises as to whether the virus provides the enzyme for the terminal step in the synthesis of the deoxyriboside of hydroxymethylcytosine or whether the host contains an enzyme which is not normally functioning to produce significant amounts of this compound. Preliminary data suggest that neither the pyrimidine nor its deoxyriboside is a normal intermediate in pyrimidine metabolism (Cohen, unpublished) and the mechanism which permits the new compound to be produced during some types of virus growth is a subject for continued investigation.

To what extent can this special pyrimidine contribute to an explanation of the changes in nucleic acid metabolism observed during virus multiplication? A reorganization of DNA synthesis appears to be a general phenomenon in virus-infected bacteria, and cannot depend on any unusual pyrimidine base. Total inhibition of RNA synthesis, however, has as yet been clearly demonstrated only with phages T 2, T 4 and T 6, and may possibly be related to their content of hydroxymethylcytosine. If this substance, or the virus containing it, were to block, in the above scheme, either the production or the utilization of cytosine, a shunt of all nucleic acid synthesis into production of virus DNA, as is observed, would be the result. Just how such inhibition might be caused, however, we cannot at present say.

We have also noted (unpublished experiments) that the DNA of these viruses is more resistant to the action of deoxyribonuclease and phosphatase than is thymus DNA. This may possibly give the virus nucleic acid a selective advantage in the infected cell.

Finally, we may note that the presence of a different pyrimidine base provides a marker by which virus DNA may be distinguished from host DNA. This will be of use in determining, for example, whether virus DNA or its components are being synthesized during the first few minutes of infection of *Esch. coli* B by T 2, when the net DNA content of the cell remains constant.

SUMMARY

1. The deoxyribonucleic acids of bacteriophages T 2, T 4 and T 6 of *Esch. coli* contain no cytosine. Instead, they contain a hitherto unrecognized pyrimidine which has been isolated in crystalline form and found to be identical with synthetic 5-hydroxymethylcytosine.

2. The ultraviolet-absorption characteristics of 5-hydroxymethylcytosine are described, and those of its deamination product, 5-hydroxymethyluracil, partially described.

3. 5-Hydroxymethylcytosine is lost during hydrolysis of deoxyribonucleic acid with concentrated perchloric acid at 100°, but can be recovered almost quantitatively after hydrolysis with formic acid at 175°, provided that a sufficient volume is used. Yields of other bases, especially guanine, are also somewhat affected by the proportion of formic acid used.

4. The quantitative purine and pyrimidine com-

position of deoxyribonucleic acid from phages T2, T4 and T6, each in r and r^+ mutants, has been determined, and no differences could be detected among these viruses. The molar ratios adenine/thymine and guanine/5-hydroxymethylcytosine are close to unity and the ratio (adenine + thymine)/(guanine + 5-hydroxymethylcytosine) is 1·8.

5. No 5-hydroxymethylcytosine could be detected in any of the following materials: dried cells of *Esch. coli*, ox-spleen deoxyribonucleic acid, phages T5 and T7, an insect polyhedral virus, vaccinia virus and meningo-pneumonitis virus. Quantitative analyses of deoxyribonucleic acid bases from phage T5 and from vaccinia virus are reported.

6. The possible significance of 5-hydroxymethylcytosine, which may be a component peculiar to certain viruses, is discussed in relation to the metabolism of pyrimidine bases and of nucleic acids during virus infection.

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REFERENCES

Bergold, G. (1947). *Z. Naturf.* **2**b, 122.
Bergold, G. H. (1953). *Advanc. Virus Res.* **1**, 91.
Bergold, G. & Pister, L. (1948). *Z. Naturf.* **3**b, 406.
Chargaff, E. (1951). *Fed. Proc.* **10**, 654.
Chargaff, E., Lipshitz, R., Green, C. & Hodes, M. E. (1951). *J. biol. Chem.* **192**, 223.
Cohen, S. S. (1947). *Cold Spr. Harb. Symp. quant. Biol.* **12**, 35.
Cohen, S. S. (1951). *Bact. Rev.* **15**, 131.
Cohen, S. S. & Arbogast, R. (1950*a*). *J. exp. Med.* **91**, 607.
Cohen, S. S. & Arbogast, R. (1950*b*). *J. exp. Med.* **91**, 619.
Davidson, D. & Baudisch, O. (1925). *J. biol. Chem.* **64**, 619.
Elwyn, D. & Sprinson, D. B. (1950). *J. Amer. chem. Soc.* **72**, 3317.
Goldthwait, D. A. & Bendich, A. (1952). *J. biol. Chem.* **196**, 841.
Herriott, R. M. & Barlow, J. L. (1952). *J. gen. Physiol.* **36**, 17.
Hershey, A. D. & Chase, M. (1952). *J. gen. Physiol.* **36**, 39.
Hitchings, G. H., Elion, G. B., Falco, E. A. & Russell, P. B. (1949). *J. biol. Chem.* **177**, 357.
Hoagland, C. L., Smadel, J. E. & Rivers, T. M. (1940). *J. exp. Med.* **71**, 737.
Laland, S. G., Overend, W. G. & Webb, M. (1952). *J. chem. Soc.* p. 3224.
Litzinger, A. & Johnson, T. B. (1936). *J. Amer. chem. Soc.* **58**, 1936.
Markham, R. (1953). *The Nature of Virus Multiplication*, ed. P. Fildes & W. E. van Heyningen, Cambridge University Press.
Marshak, A. (1951). *Proc. nat. Acad. Sci., Wash.*, **37**, 299.
Marshak, A. & Vogel, H. J. (1951). *J. biol. Chem.* **189**, 597.
Putnam, F. W. (1952). *Exp. Cell Res. Suppl.* **2**, 345.
Reichard, P. & Estborn, B. (1951). *J. biol. Chem.* **188**, 839.
Rutten, F. J., Winkler, K. C. & de Haan, P. G. (1950). *Brit. J. exp. Path.* **31**, 369.
Siminovitch, L. & Rapkine, S. (1952). *Biochim. biophys. Acta*, **9**, 478.
Smith, J. D. & Wyatt, G. R. (1951). *Biochem. J.* **49**, 144.
Tamm, C., Hodes, M. E. & Chargaff, E. (1952). *J. biol. Chem.* **195**, 49.
Watson, J. D. & Crick, F. H. C. (1953). *Nature, Lond.*, **171**, 737.
Weed, L. L. & Cohen, S. S. (1951). *J. biol. Chem.* **192**, 693.
Wyatt, G. R. (1951*a*). *Biochem. J.* **48**, 481.
Wyatt, G. R. (1951*b*). *Biochem. J.* **48**, 484.
Wyatt, G. R. (1952*a*). *Exp. Cell Res. Suppl.* **2**, 201.
Wyatt, G. R. (1952*b*). *J. gen. Physiol.* **36**, 201.
Wyatt, G. R. & Cohen, S. S. (1952). *Nature, Lond.*, **170**, 1072.
Wyatt, G. R. & Cohen, S. S. (1953). *Ann. Inst. Pasteur*, **84**, 143.