## 270. *The Methylation of Nucleosides and Mononucleotides with Diazomethane.*

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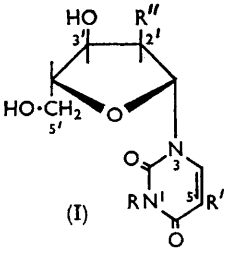
Uridine, thymidine, adenosine, 2′-deoxyadenosine, cytidine, and salts of some corresponding nucleoside 5′-phosphates have been methylated in neutral aqueous solution with ethereal diazomethane. The action of diazomethane on these substrates is compared with that of dimethyl sulphate. The significance of the results is considered in connection with the projected methylation of polynucleotides.

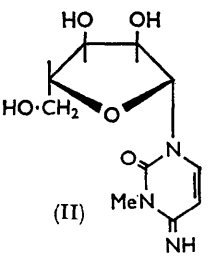
For reasons stated previously [1] we have been engaged in a systematic investigation of the action of methylating agents, and especially of diazomethane, on nucleic acid derivatives. So far, only the results of our studies on guanosine and 2′-deoxyguanosine have been reported [1] and we now wish to discuss the methylation of the other common nucleosides and of the 5′-mononucleotides derived from them.

As the ultimate object of this programme was to work with polynucleotides, it was considered important to investigate the methylation of the simpler nucleosides and nucleotides in aqueous solution. For this purpose, the technique of shaking aqueous solutions of the substrates with an excess of ethereal diazomethane was developed. A rather large excess of diazomethane was normally required as water promoted the decomposition of the reagent.

Initially, nucleosides were studied. Under the heterogeneous conditions of the reaction, uridine was rapidly and quantitatively converted by an approximately 70-molar excess of diazomethane, to 1-methyluridine (I; R = Me, R′ = H, R″ = OH), which was isolated as a pure crystalline solid. Under similar conditions but using a 100-molar excess of diazomethane, thymidine was converted into 1-methylthymidine (I; R = R′ = Me, R″ = H), obtained as a crystalline solid in 82% yield. Cytidine and adenosine both reacted more slowly and with neither was it possible to force the reaction to completion. Cytidine was 40% methylated by a 90-molar excess of diazomethane. The product,




(I)

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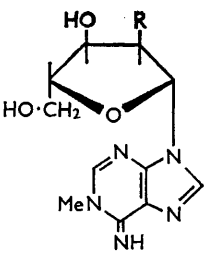



(III)

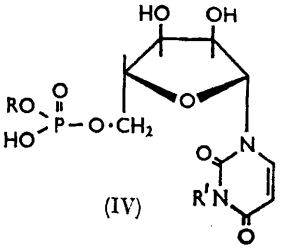
which migrated electrophoretically as a strong base, was shown, as expected from earlier work [2] with 3-methylcytosine, to be 1-methylcytidine (II) by comparison of its paper chromatographic and ultraviolet absorption spectral properties with those of authentic material, obtained by the methylation of cytidine with dimethyl sulphate.[3] Adenosine was much more resistant than cytidine to attack by diazomethane; it was approximately 10% methylated by a 400-fold excess and 30% methylated by a 800-fold excess. The main product in both cases was a strong base, identified as 1-methyladenosine (III; R = OH). However, in the 800-fold excess experiment, a small amount of 6-methylaminopurineriboside was also obtained. This might have resulted from the rearrangement

[1] Haines, Reese, and Todd, *J.*, 1962, 5281.
[2] Kenner, Reese, and Todd, *J.*, 1955, 855.
[3] Brookes and Lawley, *J.*, 1962, 1348.

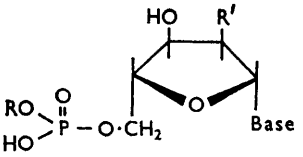
of the main product [4] (III; R = OH) although this isomerisation is very slow below pH 10. In the same way, when 2′-deoxyadenosine was treated with a large excess of diazomethane, a small yield of a strong base, assumed to be 2′-deoxy-1-methyladenosine (III; R = H), was obtained.

The above results, taken in conjunction with the previous work on guanosine,[1] suggest that the order of susceptibility to attack by diazomethane of the principal ribonucleosides is: uridine and guanosine, cytidine, adenosine. The order would be expected to be similar for deoxyribonucleosides except that thymidine is less reactive than uridine and thus probably less reactive than deoxyguanosine. The order of rate of attack by dimethyl sulphate and other methyl esters of strong acids is [5] guanosine, adenosine, and cytidine with uridine virtually inert. Thus, with the exception of guanosine, the order is reversed.

The common 5′-mononucleotides were next examined. As these compounds were monoalkyl phosphates, they were not regarded as the most suitable models for nucleic acids, which are essentially poly(dialkyl phosphates). However, it was hoped that they would provide useful information about the relative rates of the esterification and base-methylation reactions which would be relevant to the methylation of nucleic acids. Uridine-5′ phosphate (IV; R = R′ = H) was thought to be the most suitable nucleotide




(IV)




(V)

to examine first since the reaction of uridine with diazomethane was rapid and led to only one product. Aqueous solutions of sodium uridine-5′ phosphate at pH 7 were shaken with varying excess quantities of ethereal diazomethane and the products were examined by paper electrophoresis and paper chromatography, and yields were estimated spectrophotometrically. The same procedure was followed in all subsequent nucleotide methylations, and the structures of the products were deduced mainly from their electrophoretic and chromatographic properties, in some cases in conjunction with their ultraviolet absorption spectra. When a 10-fold excess of diazomethane was used, it was rapidly consumed and only 16% of the starting material (IV; R = R′ = H) remained. The three products were shown, by comparison with authentic samples, to be uridine-5′ methyl hydrogen phosphate (IV; R = Me, R′ = H) (22%), 1-methyluridine-5′ phosphate (IV; R = H, R′ = Me) (30%), and 1-methyluridine-5′ methyl hydrogen phosphate (IV; R = R′ = Me) (32%). The first of these reference compounds (IV; R = Me, R′ = H) was prepared by the esterification of uridine-5′ phosphate with methanol according to Khorana's method.[6] The second (IV; R = H, R′ = Me) was kindly provided by Mr. R. L. C. Brimacombe,[7] who prepared it by the phosphorylation of 2′,3′-*O*-isopropylidene-1-methyluridine, and the third (IV; R = R′ = Me) was prepared by treating either the first or the second with a large excess of diazomethane. This first experiment with uridine-5′ phosphate indicated that, at pH 7, N-1 was methylated nearly twice as rapidly as the phosphate group. When a 50-molar excess of diazomethane was used, all the starting material was consumed and when a 100-molar excess was used, 1-methyluridine-5′

[4] Brookes and Lawley, *J.*, 1960, 539.
[5] Lawley, *J. Chim. phys.*, 1961, 1011.
[6] Khorana, *J. Amer. Chem. Soc.*, 1959, **81**, 4657.
[7] Brimacombe, unpublished results.

methyl hydrogen phosphate (IV; R = R′ = Me) was obtained in nearly quantitative yield; only a trace of dimethyl ester was produced. This resistance to phosphate-triester formation in neutral aqueous solution was not observed in the reaction of diazomethane with nucleotides, dissolved in organic solvents.[8] Confirmation of the behaviour of neutral aqueous solutions was obtained in the preparation of 1-methyluridine-5′ methyl hydrogen phosphate (IV; R = R′ = Me) from uridine-5′ methyl hydrogen phosphate (IV; R = Me, R′ = H) using a 110-molar excess of diazomethane, only traces of dimethyl ester being formed. In the same way, uridine-2′,3′-cyclic phosphate was not esterified by a 55-molar excess of diazomethane, but was quantitatively converted into a new product (presumably its 1-methyl derivative) with the same electrophoretic mobility at pH 7·4 as the starting material. The last two compounds were dialkyl phosphates and thus suitable models for polynucleotides.

The behaviour of all the other 5′-nucleotides which were studied was, with the exception of that of guanosine-5′ phosphate, more straightforward, as the phosphate functions were methylated much more rapidly than the bases. When a neutral aqueous solution of sodium thymidine-5′ phosphate was shaken with an approximately 10-molar excess of ethereal diazomethane, the most abundant product was its methyl ester (V; Base = thymine-3, R = Me, R′ = H). Possibly the inductive effect of the 5-methyl group of thymidine was responsible for the lower acidity of its N-1 proton, relative to that of uridine. This lower acidity has been confirmed by p$K_a$ measurements.[9]

As might have been predicted from methylation studies with adenosine, when a neutral aqueous solution of adenosine-5′ phosphate was treated with diazomethane, very little base-methylation preceded monoesterification. When a 55-molar excess of diazomethane was used, the products contained starting material and methyl ester (V; Base = adenine-9, R = Me, R′ = OH) in the proportion 1 : 2. After treatment with 130-molar excess of diazomethane, only 2% starting material remained together with methyl ester (81%) and 1-methyladenosine-5′ methyl hydrogen phosphate (V; Base = 1-methyladenine-9, R = Me, R′ = OH) (17%). This was in contrast with the methylation of adenosine-5′ phosphate with dimethyl sulphate [10] where *N*-methylation was favoured over esterification, especially at pH 5.

The diazomethane methylation of cytidine-5′ phosphate also proceeded by a rapid esterification reaction accompanied by a slow attack on N-1. When a neutral aqueous solution of this nucleotide was shaken with a 60-molar excess of diazomethane, the products contained starting material (19%), its methyl ester (V; Base = cytosine-3, R = Me, R′ = OH) (69%) and 1-methylcytidine-5′ methyl hydrogen phosphate (V; Base = 1-methylcytosine-3, R = Me, R′ = OH) (12%). After treatment with 150 moles of diazomethane, no starting material remained and the products contained only the last two compounds in the proportion 2 : 1, respectively. In order to make the comparison made above for adenosine-5′ phosphate, cytidine-5′ phosphate was treated with an excess of dimethyl sulphate while the pH was maintained in the range 5—7. The products were composed of starting material (15%), its methyl ester (8%), 1-methylcytidine-5′ phosphate (V; Base = 1-methylcytosine-3, R = H, R′ = OH) (49%), and its methyl ester (28%). As was found [10] for adenosine-5′ phosphate, methylation at N-1 was favoured over esterification, and in both cases the action of dimethyl sulphate differed markedly from that of diazomethane.

Finally, and for the sake of completeness, the diazomethane methylation of guanosine-5′ phosphate was investigated. If more than a 10-fold excess of diazomethane was used, the complex reactions of the guanine residue made analysis of the products difficult. In the 10-fold excess experiment, the products contained starting material (28%), its methyl ester (33%), 7-methylguanosine-5′ phosphate (V; Base = 7-methylguanine-9, R = H,

[8] Szer and Shugar, *Acta Biochem. Polon.*, 1960, **7**, 491.
[9] Jordan, " The Chemistry of Nucleic Acids," Butterworths, London, 1960, p. 137.
[10] Griffin and Reese, *Biochim. Biophys. Acta*, 1963, **68**, 185.

R′ = OH) (23%) and its methyl ester (11%). Thus esterification was slightly more rapid than methylation at N-7.

The investigations described above not only indicate the relative susceptibilities of the bases of nucleic acids to attack by diazomethane, but they also suggest that if neutral aqueous solutions of polynucleotides were methylated, no appreciable attack on the internucleotidic linkages would occur. Thus undesirable chain fission by subsequent hydrolysis should be prevented. The nucleotides studied were mainly monoalkyl phosphates, dibasic acids with p$K_a$'s of approximately 1 and 6. At pH 7, such substances are predominantly di-anionic and only about 10% mono-anionic. As methylation by diazomethane is supposed to proceed by the substitution of a methyl group in the place of an active proton,[11] only mono-anions should react and the products should be monomethyl esters. The latter, like nucleic acids, are dialkyl phosphates which are monobasic acids with p$K \sim 1$; such species have a negligible chance of being undissociated at pH 7 so that they should be virtually resistant to esterification by diazomethane. Methylation by reagents such as dimethyl sulphate proceeds by a nucleophilic substitution reaction and thus depends on the nucleophilic character of the substrate. At pH 7, monoalkyl phosphates, such as adenosine-5′ phosphate, are largely present as comparatively nucleophilic dianions and thus monoesterification competes with methylation on N-1. At pH 4·5—5, the concentration of the di-anion is much lower and hence much less esterification occurs. The mono-anions of dialkyl phosphates would be expected to be much less nucleophilic and might therefore be inert to dimethyl sulphate. Thus the latter reagent would be expected to methylate nucleic acids only on the base residues.

In conclusion, it seems that diazomethane is a potentially useful reagent for altering some of the bases of polynucleotides without concomitant degradation, provided that reactions are carried out on neutral aqueous solutions of substrates. Diazomethane is, to some extent, complementary in its action to methyl esters of strong acids and should have interesting applications in the study of mutagenesis and in the determination of the structures of polynucleotides. It should be noted, however, that when the latter possess secondary structure, some of their base-residues would be expected to be more resistant to the attack of diazomethane than they are in the simple monomers described above.

## Experimental

Ultraviolet absorption spectra were measured on a Cary recording spectrophotometer, model 14M-50. Paper electrophoresis on Whatman No. 1 paper was conducted in a carbon tetrachloride-cooled apparatus (33v/cm.) with the following buffers: 0·1M-acetate, pH 4·5; 0·06M-phosphate, pH 5·6; 0·06M-phosphate, pH 7·4; 0·05M-triethylammonium hydrogen carbonate, pH 8; 0·1M-sodium carbonate, pH 10·5. Whatman No. 1 paper was used for ascending chromatography in the following systems: A, propan-2-ol–ammonia (*d* 0·88)–water (7,1,2); B, butan-1-ol saturated with water; C, ethanol–ammonia (*d* 0·88)–water (80,2,12); D, methanol–concentrated hydrochloric acid–water (7,2,1); E, butan-1-ol–acetic acid–water (5,2,3); F, saturated aqueous ammonium sulphate–propan-2-ol–0·1M-phosphate buffer, pH 7·2 (79,2,19); G, ethanol–M-ammonium acetate (5,2). Strong, medium, and weak chromatographic spots are represented by the letters s, m, and w, respectively.

*Methylations of Aqueous Solutions of Substrates with Ethereal Diazomethane.*—Ethereal diazomethane was estimated by titration with standardised benzoic acid. Aqueous solutions of substrates and varying excesses of ethereal diazomethane (1 c.c. $\sim 5{\cdot}5 \times 10^{-4}$ mole) were shaken together at 0° in vessels, closed by Bunsen valves. Diazomethane was consumed very rapidly. In preliminary experiments, ethereal diazomethane (10 c.c., $5 \times 10^{-3}$ mole) was shaken with (*a*) water (5 c.c.), (*b*) adenosine ($5 \times 10^{-5}$ mole) in water (5 c.c.), (*c*) uridine ($5 \times 10^{-5}$ mole) in water (5 c.c.). The respective times for total consumption of diazomethane were 100, 90, and 45 sec. Adenosine remained virtually unchanged whilst uridine was completely converted into 1-methyluridine (I; R = Me, R′ = H, R″ = OH).

[11] Eistert, *Angew. Chem.*, 1941, **54**, 99.

*Methylation of Uridine.*—A solution of uridine (0·15 g.) in water (20 c.c.) was shaken with ethereal diazomethane (75 c.c., 70 mol.). The aqueous layer was separated from the colourless ether layer and lyophilised to yield a colourless, viscous oil (0·16 g.) which crystallised from methanol–ethyl acetate–ether as needles, m. p. 115—116° (Found: C, 46·4; H, 5·6; N, 10·9. Calc. for $C_{10}H_{14}N_2O_6$: C, 46·5; H, 5·5; N, 10·9%) [lit.,[12] m. p. for 1-methyluridine (I; R = Me, R′ = H, R″ = OH), 122—123°]; $R_F$ 0·70 (system A), 0·39 (system B); paper electrophoresis (pH 10·5): neutral, periodate-consuming species.

*Methylation of Thymidine.*—A solution of thymidine (0·122 g.) in water (20 c.c.) was shaken with ethereal diazomethane (90 c.c., 100 mol.) until the diazomethane had been consumed. The separated aqueous layer was lyophilised and the colourless product crystallised from water. It (0·104 g., 82%) had m. p. 128·5—132° (Found: C, 51·7; H, 6·5. Calc. for $C_{11}H_{16}N_2O_5$: C, 51·6; H, 6·3%) [lit.,[13] m. p. for 1-methylthymidine (I; R = R′ = Me, R″ = H), 129—131°]; $R_F$ 0·87 (system A); paper electrophoresis (pH 10·5): zero mobility.

*Methylation of Cytidine.*—Cytidine (22·5 mg.) in aqueous solution (10 c.c.) was shaken with ethereal diazomethane (15 c.c., 90 mol.). Paper electrophoresis (pH 4·5) indicated the presence of ~40% of a strongly basic product with the same migration as authentic 1-methylcytidine [3] (II). Like the latter compound, this product had $R_F$ 0·64 (system C), 0·69 (system D) and had at pH, 1 $\lambda_{max.}$ 277, $\lambda_{min.}$ 243 mμ ($\varepsilon_{280}/\varepsilon_{260}$ 1·6); at pH 13, $\lambda_{max.}$ 267, $\lambda_{min.}$ 244 mμ ($\varepsilon_{280}/\varepsilon_{260}$ 0·78).

*Methylation of Adenosine.*—(*a*) Adenosine (10 mg.) in aqueous solution (4 c.c.) was shaken with ethereal diazomethane (5 × 5 c.c., 400 mol.). Paper electrophoresis (pH 4·5) indicated a strongly basic product (~10%) with the same mobility as 1-methyladenosine (III; R = OH), prepared by the action of dimethyl sulphate on adenosine. Like 1-methyladenosine, this product had $R_F$ 0·11 (system B), 0·50 (system E), 0·64 (system F), and had at pH 1, $\lambda_{max.}$ 257, $\lambda_{min.}$ 230 mμ ($\varepsilon_{max.}/\varepsilon_{min.}$ 3·7); at pH 7, $\lambda_{max.}$ 259, $\lambda_{min.}$ 233 mμ ($\varepsilon_{max.}/\varepsilon_{min.}$ 3·6). (*b*) This experiment differed from (*a*) in that twice the quantity of ethereal diazomethane (10 × 5 c.c., 800 mol.) was used. Two products were detected: 1-methyladenosine (~30%) and a more weakly basic material (~5%) with $R_F$ 0·44 (system B), 0·72 (system E). The latter had the same chromatographic properties as an authentic specimen of 6-methylaminopurineriboside, kindly provided by Dr. Beverly Griffin.

*Methylation of 2′-Deoxyadenosine.*—Treatment of 2′-deoxyadenosine (11 mg.) in water (5 c.c.) with ethereal diazomethane (2 × 5 c.c., 180 mol.) gave a low yield of a strongly basic product, as indicated by paper electrophoresis (pH 4·5). It had $R_F$ 0·08 (system B); it was assumed to be 2′-deoxy-1-methyladenosine (III; R = H).

*Methylation of Uridine-5′ Phosphate.*—(*a*) A solution of disodium uridine-5′ phosphate (4 mg.) in water (0·4 c.c.) was shaken with ethereal diazomethane (0·2 c.c., 10 mol.). Paper electrophoresis (pH 10·5) indicated starting material and three less mobile products, which migrated towards the anode with mobilities corresponding to those of (iii), (ii), and (iv) respectively; paper electrophoresis (pH 8) indicated starting material and one less-mobile anionic fraction. Paper chromatography revealed (i) starting material, $R_F$ 0·03 (system A), 0·65 (system F); (ii) uridine-5′ methyl hydrogen phosphate (IV; R = Me, R′ = H), $R_F$ 0·22 (system A), 0·59 (system F); (iii) 1-methyluridine-5′ phosphate (IV; R = H, R′ = Me), $R_F$ 0·11 (system A), 0·54 (system F); and (iv) 1-methyluridine-5′ methyl hydrogen phosphate (IV; R = R′ = Me), $R_F$ 0·52 (system A). Marker spots were used to identify all products: (ii) was prepared from methanol and uridine-5′ phosphate by Khorana's method; [6] (iii) was kindly provided by Mr. R. L. C. Brimacombe [7]; (iv) was prepared as described below.

The products were separated by paper chromatography (system A), and each of the resolved bands was eluted with 0·1N-hydrochloric acid (5 c.c.). The optical densities (at 260 mμ) were (i) 0·36 (16%), (ii) 0·48 (22%), (iii) 0·67 (30%), and (iv) 0·72 (32%). It was assumed that the products had the same extinction coefficients as the corresponding parent nucleosides (*i.e.*, uridine and 1-methyluridine) at 260 mμ, and the calculated percentages of the four constituents are indicated in parentheses.

(*b*) A solution of disodium uridine-5′ phosphate (19·5 mg.) in water (2 c.c.) was shaken with ethereal diazomethane (5 c.c., 50 mol.). Paper chromatography (system A) indicated no starting material but three products with $R_F$'s: 0·17s, 0·26w, and 0·52s. When twice the amount of ethereal diazomethane (2 × 5 c.c., 100 mol.) was used, the products had $R_F$'s (system

[12] Visser, Barron, and Beltz, *J. Amer. Chem. Soc.*, 1953, **75**, 2017.
[13] Beltz and Visser, *J. Amer. Chem. Soc.*, 1955, **77**, 736.

A): 0·17w, 0·52s, and 0·62w. $R_F$'s of reference compounds 1-methyluridine-5′ phosphate (IV; R = H, R′ = Me), uridine-5′ methyl hydrogen phosphate (IV; R = Me, R′ = H), and 1-methyluridine-5′ methyl hydrogen phosphate were 0·17, 0·26, and 0·52, respectively. The trace of material with $R_F$ 0·62 in the last experiment was assumed to be 1-methyluridine-5′ dimethyl phosphate.

*Methylation of Uridine-5′ Methyl Hydrogen Phosphate* (IV; R = Me, R′ = H).—A solution of the pyridinium salt of uridine-5′ methyl hydrogen phosphate (3·8 mg.) in water (2 c.c.) was shaken with ethereal diazomethane (2 c.c., 110 mol.). Paper electrophoresis (pH 10·5) indicated only the presence of a mono-dissociated species; paper electrophoresis (pH 8) only indicated a fraction with the same mobility as the starting material, $R_F$ (system A) 0·52. This product was identical with the methylation product of 1-methyluridine-5′ phosphate (see below), *i.e.*, 1-methyluridine-5′ methyl hydrogen phosphate (IV; R = R′ = Me).

*Methylation of 1-Methyluridine-5′ Phosphate* (IV; R = H, R′ = Me).—A solution of barium 1-methyluridine-5′ phosphate (4 mg.) in water (0·4 c.c.) was shaken with ethereal diazomethane (0·5 c.c., 35 mol.). Paper electrophoresis (both at pH 8 and 10·5) indicated starting material and a less mobile anionic fraction. Paper chromatography revealed approximately equal quantities of starting material, $R_F$ 0·11 (system A), 0·33 (system E), 0·62 (system F), and one product, $R_F$ 0·52 (system A), 0·38 (system E), 0·51 (system F), which was identical with the methylation product of uridine-5′ methyl hydrogen phosphate (see above).

*Methylation of Uridine-2′,3′-cyclic Phosphate.*—A solution of barium uridine-2′,3′-cyclic phosphate (9 mg.) in water (1 c.c.) was shaken with ethereal diazomethane (2 × 1 c.c., 55 mol.). Paper electrophoresis (pH 7·4) indicated only mono-dissociated products. Paper chromatography (system G) revealed only one material, $R_F$ 0·67 ($R_F$ of starting material, 0·50); this was presumably 1-methyluridine-2′,3′-cyclic phosphate.

*Methylation of Thymidine-5′ Phosphate.*—A neutralised (with 3N-aqueous sodium hydroxide) solution of thymidine-5′ phosphate, obtained by passing a solution of the diammonium salt dihydrate (25 mg.) through a Dowex 50 column ($H^+$ form; 3 cm. × 1 cm.$^2$), was concentrated (to 2 c.c.) and shaken with ethereal diazomethane (1 c.c., 10 mol.). Paper electrophoresis (pH 8) separated the products into (i) a fast-running and (ii) a slow-running anionic fraction. Both fractions were examined paper chromatographically (system A): (i) was resolved into two substances with $R_F$'s 0·09s and 0·60w, the former had the same $R_F$ as the starting material and thus the latter was assumed to be 1-methylthymidine-5′ phosphate (V; Base = 1-methylthymine-3, R = R′ = H); (ii) was resolved into two substances with $R_F$'s 0·47 and 0·72, the former had the same $R_F$ as thymidine-5′ methyl hydrogen phosphate (V; Base = thymine-3, R = Me, R′ = H) and the latter, which could be prepared by methylation of the former (see below), as assumed to be 1-methylthymidine-5′ methyl hydrogen phosphate (V; Base = 1-methylthymine-3, R = Me, R′ = H).

A chromatogram (system A) of the products was developed and each of the four bands obtained was eluted with N-hydrochloric acid (10 c.c.). Optical-density measurements (267 mμ) suggested that the products contained starting material (48%), its methyl ester (42%), 1-methylthymidine-5′ phosphate (1%), and its methyl ester (9%). When a 50-molar excess of ethereal diazomethane (5 × 1 c.c.) was used, paper chromatography (system A) indicated that all the starting material had been consumed.

*Methylation of Thymidine-5′ Methyl Hydrogen Phosphate* (V; Base = Thymine-3, R = Me, R′ = H).—A solution of the pyridinium salt of thymidine-5′ methyl hydrogen phosphate (7 mg.) in water (1 c.c.) was shaken with ethereal diazomethane (3·5 c.c., 115 mol.). Paper electrophoresis (pH 10·5) revealed a single anionic fraction, less mobile than starting material. Paper chromatography (system A) showed that starting material ($R_F$ 0·54) had been consumed and that only one product ($R_F$ 0·75), assumed to be 1-methylthymidine-5′ methyl hydrogen phosphate, was obtained.

*Methylation of Cytidine-5′ Phosphate.*—(a) *With diazomethane.* A solution of cytidine-5′ phosphate (3 mg.) in water (2 c.c.) was neutralised with 3N-aqueous sodium hydroxide and shaken with ethereal diazomethane (2 × 0·5 c.c., 60 mol.). Paper electrophoresis (pH 8) indicated (i) starting material, (ii) a less mobile anionic species (at pH 12, $\lambda_{max.}$ 271, $\lambda_{min.}$ 252, $\lambda_{infl.}$ 229 mμ), and (iii) a non-mobile material (at pH 12, $\lambda_{max.}$ 266, $\lambda_{min.}$ 245 mμ). The last two compounds were assumed to be cytidine-5′ methyl hydrogen phosphate (V; Base = cytosine-3, R = Me, R′ = OH) and 1-methylcytidine-5′ methyl hydrogen phosphate (V; Base = 1-methylcytosine-3, R = Me, R′ = OH), respectively. Paper electrophoresis (pH 4·5) indicated an

anionic and a neutral fraction. A paper electrophoretogram (pH 8) was developed and each of the three bands was eluted with 0·1N-hydrochloric acid (5 c.c.). The percentage compositions of the constituents (i), (ii), and (iii) were estimated to be 19, 69, and 12, respectively, by optical density measurements (at 277 mμ) of the eluates.

A neutralised solution of cytidine-5′ phosphate (18 mg.) in water (10 c.c.) was shaken with ethereal diazomethane (3 × 5 c.c., 150 mol.). Paper chromatography (system A) indicated that all the starting material (i) ($R_F$ 0·10) had been consumed, and that products (ii) ($R_F$ 0·49) and (iii) ($R_F$ 0·67) were present in the proportion 2 : 1.

(b) *With dimethyl sulphate.* A solution of cytidine-5′ phosphate (0·092 g.) in water (3 c.c.), to which had been added 3N-sodium hydroxide (to pH 5), was magnetically stirred while dimethyl sulphate (0·2 g., 5·6 mol.) was added slowly during 1 hr. The pH was kept in the range 5—7 by the gradual addition of 3N-sodium hydroxide. Paper electrophoresis (pH 5·6) resolved the products into an anionic and a neutral fraction. Both fractions were eluted and resubmitted to paper electrophoresis (pH 7·4). The anionic fraction was resolved into (i) a faster-running material with the same mobility as cytidine-5′ phosphate and (ii) a slower-running material assumed to be cytidine-5′ methyl hydrogen phosphate. The neutral fraction was resolved into (iii) a neutral material, 1-methylcytidine-5′ methyl hydrogen phosphate, and (iv) and anionic material, not observed in the diazomethane experiment and assumed to be 1-methylcytidine-5′ phosphate (V; Base = 1-methylcytosine-3, R = H, R′ = OH). $R_F$'s (system A) were (i) 0·10, (ii) 0·49, (iii) 0·67, and (iv) 0·19.

The products were separated by paper electrophoresis (pH 7·4) and the four bands were eluted and estimated as above. The percentage composition of the constituents was found to be (i) 15, (ii) 8, (iii) 28, and (iv) 49.

*Methylation of Adenosine-5′ Phosphate.*—(*a*) A solution of adenosine-5′ phosphate (18 mg.) in water (10 c.c.), neutralised with 3N-sodium hydroxide, was shaken with ethereal diazomethane (5 c.c., 55 mol.). Paper electrophoresis (pH 8) indicated (i) starting material and (ii) a less mobile anionic species, assumed to be adenosine-5′ methyl hydrogen phosphate (V; Base = adenine-9, R = Me, R′ = OH) in the ratio 1 : 2. Paper electrophoresis (pH 4·5) indicated only one fraction with the same mobility as starting material.

(*b*) A neutralised solution of adenosine-5′ phosphate (3·2 mg.) in water (2 c.c.) was shaken with ethereal diazomethane (2 c.c., 130 mol.). Paper electrophoresis (pH 8) resolved the products into three bands: (i) a faster-running anionic species, identified as starting material; (ii) a slower-running anionic species, assumed to be the methyl ester of (i); and (iii) a neutral species assumed to be 1-methyladenosine-5′ methyl hydrogen phosphate (V; Base = 1-methyladenine-9, R = Me, R′ = OH). Each of the three bands was eluted with 0·1N-hydrochloric acid (5 c.c.) and the optical densities (at 257 mμ) of their solutions were measured. The percentage composition of the products was found to be (i) 2, (ii) 81, and (iii) 17. $R_F$'s (systems A and F, respectively) were (i) 0·16, 0·35; (ii) 0·35, 0·26; and (iii) 0·60, 0·61.

*Methylation of Guanosine-5′ Phosphate.*—A solution of disodium guanosine-5′ phosphate (20·5 mg.) in water (10 c.c.) was shaken with ethereal diazomethane (1 c.c., 10 mol.). The products were examined by paper chromatography (system F) and four main components were observed: (i) $R_F$ 0·52, starting material, (ii) $R_F$ 0·43, the methyl ester of starting material, (iii) $R_F$ 0·69 (fluorescent in ultraviolet light), assumed to be 7-methylguanosine-5′ phosphate (V; Base = 7-methylguanine-9, R = H, R′ = OH), and (iv) $R_F$ 0·63 (fluorescent in ultraviolet light), assumed to be the methyl ester of (iii). The products were separated by paper electrophoresis (pH 8) into four anionic bands of decreasing mobility (i), (iii) fluorescent, (ii), and (iv) fluorescent. Each band was eluted with water (5 c.c.) and the optical densities were measured at $\lambda_{max.}$. The percentage composition of the products was found to be (i) 28, (ii) 33, (iii) 23, and (iv) 11. The ultraviolet absorption spectra of (iii) and (iv) corresponded with the spectrum of 7-methylguanosine.[1]

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