Nucleotides. Part XXVI. The Methylation of Uridylic Acid b.*

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Repeated methylation of pure uridylic acid *b* with Purdie's reagent gives an apparently uniform material which, when hydrogenated and submitted to acid hydrolysis, yields 2 : 5- and 3 : 5-di-*O*-methylribose and smaller amounts of 2-, 3-, and 5-*O*-methylribose. The method is therefore of no value for the orientation of uridylic acids *a* and *b*. The bearing of these results on the validity of methylation studies as applied to the structural investigation of ribonucleic acids is discussed.

In an earlier paper in this series (Part X, Brown and Todd, *J.*, 1952, 52), possible structures for the ribonucleic acids were discussed and one in which the individual nucleoside residues were linked through the 3'- and 5'-positions by phosphodiester groupings was preferred. Corresponding structures in which 2' : 5'-phosphodiester linkages, or a mixture of 2' : 5'- and 3' : 5'-linkages occurred, could not be excluded on chemical grounds at that time because of the phosphoryl migration which accompanied chemical hydrolysis of ribonucleic acids. The problem was further complicated by the fact that the products of hydrolysis—the four pairs of isomeric *a* and *b* nucleotides—had not been oriented with respect to the position of the phosphoryl group. That the *a* and the *b* nucleotides were the 2'- and the 3'-phosphates of the corresponding nucleosides was clear, but which was 2'- and which 3'- was unknown (Part IX, *idem*, *J.*, 1952, 44). Further work has shown that the ribonucleic acids are to be regarded as *b* : 5'-linked polynucleotides, *i.e.*, that the internucleotidic linkage can be defined in relation to the isolated mononucleotides (Part XIII, Brown, Dekker, and Todd, *J.*, 1952, 2715; Part XXI, Brown and Todd, *J.*, 1953, 2040; Part XXIV, Brown, Heppel, and Hilmoe, *J.*, 1954, 40). It therefore remains only to determine the true location of the phosphoryl group in the *a* and *b* nucleotides to distinguish finally between 2' : 5' and 3' : 5' as possible positions of the internucleotidic linkage in ribonucleic acids.

The classical method of structural determination in the carbohydrate series—complete methylation followed by hydrolysis and identification of the methylated sugars—is valid only in the absence of group migration, and it was therefore decided in the first instance to study the methylation of a simple nucleotide to find if the method had any value for structural determination in this field. Our interest in this problem was enhanced when Anderson, Barker, Gulland, and Lock (*J.*, 1952, 369) described the methylation of nucleosides and of yeast ribonucleic acid with Purdie's reagent. On hydrolysing the methylated nucleic acid these authors obtained, in rather poor yield, a mixture containing free ribose as well as mono- and di-methylriboses. They concluded that a large amount of chain-branching on sugar occurs in yeast ribonucleic acid, *i.e.*, that the unmethylated ribose originated in branching points where all three hydroxyl groups in a ribofuranose residue are involved in phosphodiester linkages. We had earlier suggested this type of branching on other grounds (Part X, *loc. cit.*), but in view of the known lability of ribonucleic acids or, more generally, of esters of *a* and *b* nucleotides in presence of basic reagents (Part IX, *loc. cit.*), it seemed to us that a study of the methylation of a simple nucleotide would be necessary before the validity of the conclusions of Anderson *et al.* (*loc. cit.*) regarding chain-branching could be assessed.

Crystalline uridylic acid *b*, which was shown by ion-exchange chromatography to be uncontaminated by any of the *a* isomer, was repeatedly methylated with methyl iodide and silver oxide, methanol being used initially as solvent, *i.e.*, the procedure was essentially that used by Anderson *et al.* (*loc. cit.*). The course and extent of methylation at each stage was readily followed by paper-chromatographic examination, since increasing substitution gave products with increasing R_F values in the solvent systems used. In the intermediate stages of the methylation, several chromatographically distinct materials

* Part XXV, *J.*, 1954, 46.

were present, but after fourteen successive methylations the product ran as a single fast-running spot on chromatograms using various solvent systems and methylation appeared to be essentially complete. The methylated product was next hydrogenated to labilise the sugar residue (Levene and Jorpes, *J. Biol. Chem.*, 1929, **81**, 575) and then hydrolysed with 0·5N-sulphuric acid, the conditions necessary for optimum yields of methylated sugars having been determined in preliminary experiments. In addition to 4 : 5-dihydro-1-methyluracil the crude hydrolysate contained dimethylriboses, a small amount of monomethylriboses, and some acidic phosphorylated methylriboses. A neutral phosphorus-containing substance, fast-running on chromatograms (probably a dimethyl ester of dimethylribose phosphate), was also present in traces; it was evidently an intermediate in the hydrolysis since it occurred in greater amount in short hydrolyses and finally disappeared on prolonged acid treatment, yielding dimethylribose. The hydrolysate was extracted with chloroform and the extract evaporated, giving a mixture of methylated riboses contaminated with 4 : 5-dihydro-1-methyluracil and a trace of 4 : 5-dihydrouracil. This mixture was chromatographed on a cellulose column with butanol–water as solvent system; in this way four fractions were obtained, one a dimethylribose fraction and three minor monomethylribose fractions.

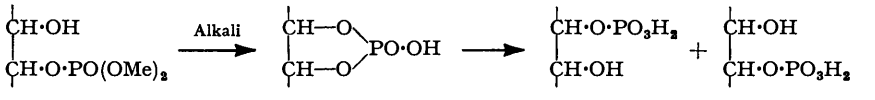
The dimethylribose fraction, which was isolated in an analytically pure state, ran as a single spot on paper chromatograms in all the solvents examined. It was clearly distinguishable from 2 : 3-di-*O*-methylribose but was demonstrably a mixture. On periodate oxidation the material took up *ca.* 1 mol. of oxidant but only 0·65—0·85 mol. of formic acid was liberated even on prolonged oxidation; this is consistent with the view that it was a mixture of 2 : 5- and 3 : 5-di-*O*-methyl-ribose, the latter being the major component. Further, on paper electrophoresis in borate buffer, two spots were observed, the more intense migrating towards the anode at a rate comparable with that of ribose, and the other, like the "inert" non-complexing 2 : 3 : 5-tri-*O*-methylribose, having zero migration. Theoretical considerations relating the structure of sugar derivatives to their electrophoretic behaviour in presence of borate (Foster, *J.*, 1953, 982) would predict precisely this behaviour for 3 : 5- and 2 : 5-di-*O*-methylribose respectively. Final confirmation of this view was obtained by the preparation of osazones from the mixture. Treatment with *p*-bromophenylhydrazine furnished two osazones which were separated and purified by chromatography. These were shown to be the *p*-bromophenylosazones of 3 : 5-di-*O*-methylribose and 5-*O*-methylribose by direct comparison with authentic samples prepared from 2 : 3 : 5-tri-*O*-methylribose (Barker, *J.*, 1948, 2035) and 5-*O*-methylribose (Levene and Stiller, *J. Biol. Chem.*, 1934, **104**, 299) respectively. Clearly the osazone of 5-*O*-methylribose was derived from 2 : 5-di-*O*-methylribose in the original material by elimination of the 2-*O*-methyl group. From a consideration of the yields of the two osazones from the mixed dimethylriboses and those obtained in preparing authentic specimens under standard conditions, it appeared that the mixture contained *ca.* 85% of 3 : 5-di-*O*-methylribose, a value similar to that deduced from periodate oxidation experiments.

Slower-running reducing spots observed on paper chromatograms of the hydrolysis mixture were due to small amounts of 2-, 3-, and 5-*O*-methylribose. These monomethyl sugars were satisfactorily separated on the cellulose column but on the scale of operation used the amounts isolated were too small to permit their complete characterisation. However, their paper chromatographic and electrophoretic behaviour leaves little doubt as to their identity. The first to be eluted from the column was identical in chromatographic and electrophoretic behaviour with authentic 5-*O*-methylribose. The other two were assigned structures on the basis of their chromatographic behaviour and the fact that, since the uridylic acid methylated has a furanose structure, neither could be 4-*O*-methylribose. When submitted to electrophoresis in presence of borate one of them migrated at a rate comparable to that of ribose and 3 : 5-di-*O*-methylribose. We therefore concluded that it was to be regarded as 3-*O*-methylribose, and have since obtained strong evidence substantiating this formulation (Part XXVII, succeeding paper). The other, like 2-deoxy-L-ribose, travelled much more slowly, and is therefore considered to be 2-*O*-methylribose. Substitution at the 2-position in a sugar leads to a considerable decrease in the rate of migration,

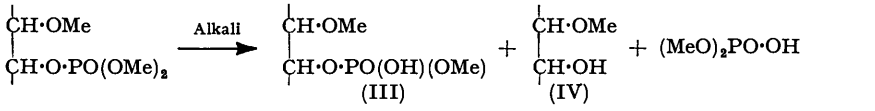
presumably because the preferred formation of a cyclic 1 : 2-*O*-borate complex is prevented and only less stable complexes involving other hydroxyl groups can be formed (Foster, *loc. cit.*; see also Foster and Stacey, *J. Appl. Chem.*, 1953, **3**, 19). It is of interest that the substance we identify as 2-*O*-methylribose appeared to be present (presumably as a result of incomplete methylation) in a sample of 2 : 3-di-*O*-methylribose prepared by methylation of *N* : 5′-dityladenosine and subsequent hydrolysis. The monomethylriboses encountered among the hydrolysis products of methylated uridylic acid *b* apparently owe their presence to incomplete methylation, since treatment of the isolated dimethylriboses with acid under the same conditions of hydrolysis did not effect any observable demethylation.

The results of our experiments show clearly that, whatever be the position of the phosphate group in uridylic acid *b*, phosphoryl migration occurs during methylation and hence that the methylation technique is of no value, at least in this form, for orienting the uridylic acids *a* and *b*; it seems reasonable to conclude that it would be equally invalid in the case of the other pairs of *a* and *b* nucleotides. That this should be so was not unexpected, since it was *a priori* likely that the initial action of the methylating agent would be to esterify the phosphoric acid grouping in the uridylic acid giving products which would undergo migration with great ease under the basic conditions of the reaction. We were able to demonstrate that this is so by subjecting uridylic acid *b* to one methylation and examining the products. In addition to unchanged starting material, a second material was present which had the chromatographic and ion-exchange characteristics of a monomethyl ester of uridylic acid. In accordance with this formulation it yielded uridylic acid on treatment with alkali; moreover, when acted upon by ribonuclease, a large proportion was hydrolysed but some remained unaffected. Since ribonuclease attacks specifically the esters of pyrimidine *b* nucleotides and not the *a* isomers (Part XXI, *loc. cit.*) it seems clear that a significant amount of migration had already occurred in one methylation. To unravel the detailed course of the repeated methylation of uridylic acid *b* would be a large undertaking and would, for the purposes of our present work, be unnecessary.

Type I



Type II



Some evidence regarding the progress of methylation was, however, obtained by studying the action of alkali on the products of methylation at various stages. If the hydroxyl group vicinal to the phosphate residue is unmethylated, *i.e.*, if the methylated material contains a grouping of Type I (above), then treatment with alkali will yield a mixture of two isomeric acids as indicated. If the vicinal hydroxyl is methylated as in Type II, then alkaline hydrolysis will cause fission either to (III) or to (IV) and dimethyl hydrogen phosphate. It was found that, as shown by paper-chromatographic studies, even after 8 successive methylations the products were mainly of Type I; on further methylation, products of Type II became predominant. It is obvious that the possibility of extensive phosphoryl migration exists throughout that part of the methylation process in which the vicinal hydroxyl group remains unmethylated.

The bearing of these findings upon the structural investigation of polynucleotides by the methylation technique (Anderson, Barker, Gulland, and Lock, *loc. cit.*) must now be considered. The structural analogy between the polynucleotides and the esters of simple mononucleotides indicates that phosphoryl migration should occur in methylation of the former as in methylation of the latter. Moreover, migration of the phosphoryl group at $C_{(2')}$ or $C_{(3')}$ would inevitably cause fission of some of the internucleotidic linkages since migration and fission are consequential on each other. Again, the difficulty we experienced in effecting complete methylation of uridylic acid is likely to be even greater in the complex

polynucleotides, so that the occurrence of appreciable amounts of ribose in the hydrolysate of the methylated nucleic acid might well be accounted for on this basis alone. Since each rupture of an internucleotidic linkage would in effect increase the number of " end-groups " capable of yielding dimethylribose, it seems clear that the observed ratios of ribose : monomethylribose : dimethylribose could be explained by a combination of these effects and need not indicate, as Anderson *et al.* concluded, a high degree of chain-branching.

In the absence of much more precise evidence of the homogeneity and molecular weight of a ribonucleic acid and of its methylation product, the results recorded in this paper suggest that the methylation technique as applied by Anderson *et al.* (*loc. cit.*) can lead to no valid conclusions regarding the degree of chain-branching in the original acid.

Experimental

Paper Chromatography and Ionophoresis.—For the paper chromatography of the substances studied the following solvent systems (top layer) were used in ascending chromatograms: A, *n*-butanol–water; B, *n*-butanol–ethanol–water–aqueous ammonia (*d* 0·880) (40 : 10 : 49 : 1, v/v); C, *n*-butanol–acetic acid–water (40 : 10 : 50, v/v); D, pyridine–ethyl acetate–water (1 : 2 : 2, v/v); E, *iso*propanol–water (70 : 30, v/v); F, *iso*propanol–aqueous ammonia (*d* 0·880)–water (70 : 5 : 25, v/v). These solvent systems are designated below by the appropriate capital letter. Nucleotides were located on paper chromatograms by photographing the dried papers in ultra-violet light; phosphates were detected by Hanes and Isherwood's reagent (*Nature*, **1949**, **164**, 1107), and sugars by Partridge's aniline hydrogen phthalate reagent (*ibid.*, p. 443). Representative R_F values are given in the Table.

Paper-chromatographic and ionophoretic characteristics of ribose derivatives.

	R_F in solvent system				M_R in 0·1M-sodium borate
	A	B	C	D	
Ribose	0·18	0·27	0·30	0·37	1·00
2-Deoxy-L-ribose	—	—	—	—	0·46
2-*O*-Methylribose	0·34	0·44	0·46	0·49	0·49
3- ,,	0·38	0·45	0·49	0·55	0·90
5- ,,	0·40	0·49	0·51	0·58	0·99
2 : 3-Di-*O*-methylribose	0·53	—	0·64	0·67	0
2 : 5- ,,	0·57	0·65	0·69	0·71	0·02
3 : 5- ,,					0·90
2 : 3 : 5-Tri-*O*-methylribose	0·68	—	—	0·84	0

Paper ionophoresis of sugar derivatives was carried out in an apparatus similar to that described by Flynn and de Mayo (*Lancet*, 1951, 235) in aqueous sodium tetraborate (0·1M) at 210 v. M_R values [equivalent to M_G values as defined by Foster and Stacey (*loc. cit.*) but with ribose and 2 : 3 : 5-tri-*O*-methylribose as reference substances] are given in the Table.

Uridylic Acid b.—The uridylic acid was prepared by acid hydrolysis of yeast ribonucleic acid, followed by ion-exchange chromatography on Dowex-2 resin (formate) (Cohn and Carter, *J. Amer. Chem. Soc.*, 1950, **72**, 2606). The gum obtained on removal of solvent gradually crystallised. After this had been stirred with methanol, *uridylic acid* b was collected and had m. p. 195° (decomp.) (Found, in material dried at 105°/0·2 mm.: C, 33·8; H, 4·5; N, 9·0. $C_9H_{13}O_9N_2P$ requires C, 33·3; H, 4·0; N, 8·7%). As far as we are aware, uridylic acid *b* has not hitherto been isolated in a crystalline state. Levene (*J. Biol. Chem.*, 1920, **41**, 1) gave m. p. 202° (corr.; decomp.) for crystalline " yeast uridylic acid."

An ion-exchange elution diagram (Part XIII, *loc. cit.*) showed only one peak, corresponding in position and optical density ratio (280/260 mμ = 0·32) to uridylic acid *b*. No trace of the *a* isomer was observed. Treatment with dilute acid converted the material into a mixture of the *a* and *b* isomers.

5-O-*Methylribose* p-*Bromophenylosazone*.—5-*O*-Methylribose (0·1 g.), *p*-bromophenylhydrazine (0·35 g., 3·1 mols.), 2N-acetic acid (3 c.c.), and Methylcellosolve (2-methoxyethanol) (1·5 c.c.) were heated together in a stoppered tube on the steam-bath for 3·5—4 hr. On cooling, the reddish oil which had separated solidified and was collected. After trituration with 2N-acetic acid and water, the solid (235 mg.) was dried and dissolved in benzene (25 c.c.). The solution was run on to a column (1·7 cm. diam.) of neutral alumina (25 g.) and washed with benzene and then with benzene containing 2% of methanol to remove dark impurities. Benzene–methanol (19 : 1) moved the product from the top of the column and after extrusion the zone containing

the osazone was extracted with hot methanol (6 × 50 c.c.). Evaporation gave yellow needles (146 mg., 54%). Recrystallisation from benzene and then methanol yielded the pure osazone in long yellow needles, m. p. 178° (Found: C, 43·5; H, 4·3; N, 11·2. Calc. for $C_{18}H_{20}O_3N_4Br_2$: C, 43·2; H, 4·0; N, 11·2%). Levene and Stiller (*loc. cit.*) give m. p. 177°.

3 : 5-*Di*-O-*methylribose* p-*Bromophenylosazone*.—This was obtained from 2 : 3 : 5-tri-*O*-methylribose (0·1 g.) under the conditions described above. The crude product (242 mg. was dissolved in boiling ether (3·5 c.c.) and filtered from acetyl-*p*-bromophenylhydrazine (21 mg.). The ethereal solution was evaporated and the residue dissolved in benzene (10 c.c.) and applied to a column of alumina (25 g.) which was washed with benzene (200 c.c.) and then benzene containing 0·5% of methanol. Benzene containing 1% of methanol (200 c.c.) then rapidly eluted the *osazone* (134 mg., 50%), which crystallised from aqueous methanol in yellow needles, m. p. 176—177°, depressed to 157—164° when mixed with 5-*O*-methylribose *p*-bromophenylosazone (Found: C, 44·8; H, 4·4; N, 11·0. $C_{19}H_{22}O_3N_4Br_2$ requires C, 44·4; H, 4·3; N, 10·9%).

5-O-*Methylribose Phenylosazone*.—The *p*-bromophenylosazones described above proved most convenient for the separation of mixtures of the parent methylated ribose derivatives. Phenylosazones could also be used but were less satisfactory.

5-O-*Methylribose phenylosazone* prepared in the usual way crystallised from methanol in yellow needles, m. p. 152° (Found: C, 62·7; H, 6·3; N, 16·5. $C_{18}H_{22}O_3N_4$ requires C, 63·1; H, 6·5; N, 16·4%).

Methylation of Uridylic Acid b.—The acid (4·0 g.) was methylated at 25—27° as described by Anderson *et al.* (*loc. cit.*) for yeast ribonucleic acid, fourteen additions of silver oxide and methyl iodide being made, with occasional filtration from silver salts and evaporation to dryness. Methanol was added in the initial stages. The product (4·9 g.) was an almost colourless thick syrup [Found, in material dried at 50°/0·2 mm. for 48 hr.: C, 41·6, 43·5; H, 6·0, 5·4; N, 6·3, 7·2; OMe, 28·4, 28·9; NMe, 4·7, 4·3. Calc. for $C_{14}H_{23}O_9N_2P$ (*i.e.*, pentamethyl derivative): C, 42·6; H, 5·9; N, 7·1; OMe, 31·5; NMe, 7·4%]. The material ran on chromatograms as one spot in all solvent systems except C, in which a trace of slightly slower-running material was observed.

Course of Methylation. At several stages during the methylation small aliquot portions were withdrawn and studied on paper chromatograms. The initial methylation product was clearly uridine methyl hydrogen phosphate (R_F 0·42 in E) (see below.) After three methylations the major components were two neutral fast-running materials (R_F 0·75, 0·84 in E), presumably nucleotide dimethyl esters. When run on chromatograms in solvent system F the whole product from three methylations was converted into material with R_F values 0·35, 0·43, 0·59 (uridine methyl hydrogen phosphate had R_F 0·35 in F). Treatment with 0·5N-sodium hydroxide at 37° overnight largely converted the neutral substance into a material with R_F similar to that of uridylic acid (0·07). This behaviour was still predominant in the product of 8 successive methylations. Thereafter the major component was not affected by the ammoniacal solvent F. The final methylation product (R_F 0·86, 0·84 in C and F respectively), when treated with sodium hydroxide, gave spots (R_F 0·79, 0·43, 0·33 in C; 0·79, 0·65 in F) together with a phosphorus-containing spot showing no ultra-violet absorption (R_F 0·25 in C, 0·51 in F) (dimethyl hydrogen phosphate ?). Phosphorus could not be detected on chromatograms of the highly methylated product by the spray reagent, except after hydrolysis by acid or alkali.

A small sample of uridylic acid *b* was methylated once (as above) and an aqueous solution of the product extracted with chloroform to remove neutral esters. Paper chromatography of the neutralised solution showed that about 40% of a substance having the R_F expected for a monomethyl ester was present. The substance was eluted from a Dowex-2 resin column (280/260 mμ = 0·30) before uridylic acid and uridine-2′ : 3′ phosphate (Part XIII, *loc. cit.*). It was completely converted into uridylic acid by alkali and partly by exhaustive treatment with ribonuclease.

Hydrogenation of Methylated Uridylic Acid b.—Hydrogenation was sluggish but was effected by platinum oxide and hydrogen (Anderson *et al.*, *loc. cit.*) in (*a*) slightly acid aqueous ethanol at atmospheric pressure and 30—50° or (*b*) in the same solvent under 4—5 atm. at room temperature. Hydrogenation was taken to be complete when there was no further decrease in absorption at 260 mμ. The product was a colourless viscous syrup.

Hydrolysis of Hydrogenated Methylation Product.—After many trial hydrolyses, the following conditions were established as affording a maximum yield of dimethylribose. In shorter hydrolyses a substance (R_F 0·80 in C) was observed which gave a positive sugar reaction but a negative phosphate reaction on paper. Phosphorus could, however, be detected in an eluate of

the spot. This material gave rise to dimethylribose and acidic methylated ribose phosphates on further hydrolysis, and was considered to be a dimethyl ester of dimethylribose phosphate.

A solution of the methylated material (2·0 g.) in 0·5N-sulphuric acid (40 c.c.) was heated on the steam-bath for 15 hr. After neutralisation of the product with barium hydroxide solution, barium sulphate was removed, the solution evaporated under reduced pressure, and the residue (2·14 g.) dried at 30—40°/0·1 mm. Extraction with boiling chloroform (3 × 35 c.c.) left an insoluble residue of barium salts of organic phosphates (0·51 g.) which were not further studied. The chloroform extract was evaporated, yielding a reddish-brown syrup (1·64 g.). This material was dissolved in *n*-butanol saturated with water (total, 15 c.c.) and applied to a column (66 × 6 cm.) of cellulose (630 g. of 90-mesh) and eluted with the same solvent (*ca.* 3 l.). Fractions (19 c.c.) were collected at the rate of 80 c.c./hr. and tested for reducing material by spotting them on filter paper and development with the sugar reagent. The exact composition of " key " fractions was ascertained by paper chromatography. Tubes containing the same material were bulked, giving 5 fractions which were evaporated to dryness under reduced pressure. They were examined in order of elution. Initial chromatographic examination showed that no ketoses were present (Partridge and Westall, *Biochem. J.*, 1948, **42**, 238).

Fraction 1. Crystallisation of the semi-solid material (0·5 g.) from ethanol or butanol yielded 4 : 5-dihydro-1-methyluracil in stout colourless needles, m. p. 129°. Anderson *et al.* (*loc. cit.*) give m. p. 129°. In addition to this material, the substance considered to be di-*O*-methylribose dimethyl phosphate was present in traces.

Fraction 2 (*dimethylribose fraction*). The pale yellow gum (185 mg.) was dissolved in a little chloroform, filtered, and evaporated, then taken up in water, filtered, evaporated, and dried to constant weight. The product was a viscous syrup (170 mg.) (Found : C, 47·5; H, 7·6. Calc. for $C_7H_{14}O_5$: C, 47·2; H, 7·9%). A further 25 mg. were obtained by evaporation of adjoining fractions.

The material ran as a compact spot on paper chromatograms with R_F different from that of 2 : 3-di-*O*-methylribose. Ionophoresis in borate buffer gave two spots corresponding to 2 : 5- (weak) and 3 : 5-di-*O*-methylribose (strong) (see Theoretical section).

Quantitative periodate oxidation and formic acid estimation gave the following figures : 26 hr., 0·78 mol./mol. of oxidant consumed; 65 hr., 0·97 (0·66 mol. of formic acid); 288 hr., 1·28 (0·85) indicating that the 3 : 5-isomer predominated. It is of interest that no formic acid could be detected on chromatograms of the dimethylribose fraction by the periodate spray technique of Buchanan, Dekker, and Long (*J.*, 1950, 3162).

No appreciable demethylation occurred when the material was heated at 100° for 15 hr. in 0·5N-sulphuric acid.

A portion of the syrup (160 mg.) was treated with *p*-bromophenylhydrazine as described above for the preparation of *p*-bromophenylosazones from 5-*O*-methyl- and 2 : 3 : 5-tri-*O*-methyl-ribose. The crude product, a yellow powder (320 mg.), was chromatographed in benzene on neutral alumina. Development with benzene containing increasing amounts of methanol (cf. above) yielded two pure products. The first eluted (216·5 mg.) was recrystallised from aqueous methanol and had m. p. 174—175° (155 mg.) undepressed in admixture with authentic 3 : 5-di-*O*-methylribose *p*-bromophenylosazone. The second, obtained in much smaller amount (30 mg.), had m. p. 177—178° after recrystallisation and was identified as 5-*O*-methylribose *p*-bromophenylosazone by m. p. and mixed m. p.

Fraction 3. The yellowish syrup (7 mg.) was examined by paper chromatography. In all solvents examined it had R_F identical with that of 5-*O*-methylribose, and it was identical with this sugar in ionophoretic behaviour (M_R value).

Fraction 4. The semi-solid material (33 mg.) was extracted with chloroform, leaving an insoluble residue which, when sublimed at 200°/0·1 mm., gave colourless crystals of 4 : 5-dihydrouracil m. p. 274°. (Traces of this substance were also present in Fractions 3 and 5.) The chloroform extract gave a yellowish syrup (19 mg.) on evaporation. On the basis of its chromatographic and ionophoretic behaviour it was identified as 3-*O*-methylribose.

Fraction 5. Extraction with boiling chloroform yielded a syrup (20 mg.) which it was concluded was essentially 2-*O*-methylribose. Its ionophoretic mobility was the same as that of 2-deoxy-L-ribose, and of another sugar present in hydrolysates of partially methylated *N* : 5′-dityrosinadenosine (cf. Theoretical section).

On paper chromatograms, satisfactory separation of mixtures of Fractions 3, 4, and 5 could only be achieved by using solvent system D.

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