The Constitution of Yeast Ribonucleic Acid. Part XVI. The Nature of Methylated Yeast Ribonucleic Acid and its Stability to Alkalis.*

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Previous evidence for the existence of a branched structure in yeast ribonucleic acid has been re-examined and earlier conclusions are withdrawn. Evidence is presented in support of current views regarding the alkaline hydrolysis of ribopolynucleotides.

A previous communication (Anderson, Barker, Gulland, and Lock, *J.*, 1952, 369) described the methylation of yeast ribonucleic acid and the hydrolysis of the product to yield ribose and mono- and di-*O*-methylriboses. On the basis of these results a structure was put forward involving branching of the polynucleotide chain, but it was pointed out that the validity of the conclusions depended on three assumptions: first, that the unsubstituted ribose does not arise through failure to methylate all available hydroxyl groups; secondly, that dimethylriboses are not produced from simple nucleotides formed by degradation of the polynucleotide; thirdly, that the mixture of carbohydrates analysed is representative of the carbohydrate residues in the methylated polynucleotide. Almost simultaneously with the publication of this work, Brown and Todd (*J.*, 1952, 52) put forward an explanation of the alkaline lability of ribopolynucleotides which materially affected the reliability of conclusions drawn from methylation experiments. Considerable discussion of the question

* Part XV, *J.*, 1952, 369.

of branching has since taken place (Brown and Todd, *loc. cit.*; Brown, Magrath, and Todd, *J.*, 1954, 1442; Markham and Smith, *Biochem. J.*, 1952, **52**, 558; Cohn, Doherty, and Volkin in "Phosphorous Metabolism, Vol. II," The Johns Hopkins Press, 1952, p. 345; Cohn and Volkin, *J. Biol. Chem.*, 1953, **203**, 319) and further work has therefore been carried out to test the previous conclusions.

Methylation of adenosine-3′ phosphate by the technique previously described gave a product which, after hydrolysis with acid, yielded ribose, mono-, di-, and tri-*O*-methylriboses and a considerable quantity of material which remained on the starting line in paper chromatograms. The last fraction, which probably consists of sugar phosphate derivatives, will be referred to later. The mono- and di-*O*-methylribose fractions were examined by paper chromatography in butanol–boric acid (Barker and Smith, *Chem. and Ind.*, 1954, 19; Barker, Noone, Smith, and Spoors, *J.*, 1955, 1327) and were found not to be homogeneous. The mono-*O*-methylribose fraction was most convenient for study and was shown to consist of 2-, 3-, and 5-*O*-methyl-D-ribose. These results were in apparent agreement with those obtained by Brown, Magrath, and Todd (*loc. cit.*) for the methylation of uridine-3′ phosphate. However, since tri-*O*-methylribose was present, it was clear that dephosphorylation had occurred, and therefore it cannot be concluded from the heterogeneity of the methylated riboses that migration of the phosphate group had taken place during methylation. The methylated adenylic acid was therefore subjected to a brief treatment with aqueous alkali and that part of the methylated product which had suffered dephosphorylation was removed by chromatography on Dowex-1 resin. That part which still retained the phosphoryl residue was hydrolysed with acid and the methylated riboses were examined as before and again found not to be homogeneous. This indicates conclusively that the phosphate ester group migrates during the methylation. It follows that in these circumstances methylation cannot distinguish between a 2′- and a 3′-phosphate ester grouping. It is also clear that, since methylation of adenylic acid is incomplete and is accompanied by some dephosphorylation, the first two assumptions quoted above require further consideration.

In the previous work, the extent of the methylation was assessed on the basis of the methoxyl content of the product, and in view of the fact that nucleosides were readily methylated, it was considered that methylation of the nucleic acid was substantially complete. It must be borne in mind, however, that the observed methoxyl content could have arisen by some residues being undermethylated and others overmethylated after degradation. In view of the results now reported with adenylic acid, the presence of ribose in the hydrolysate of methylated nucleic acid does not necessarily indicate branching of the polynucleotide chain.

The degree to which degradation occurs during the methylation of a polynucleotide is more difficult to assess than in the case of a simple nucleotide. Further, no simple method exists for separating degraded and undegraded material such as was used with methylated adenylic acid. In the previous experiments, the crude methylated nucleic acid was fractionated by solvent precipitation and the diffusion coefficient of the isolated material was compared with that of the original nucleic acid. This gives an average value for the molecular size and since the yeast ribonucleic acid used as starting material was undoubtedly polydisperse, the question of the homogeneity of the product does not merit consideration. As far as is known, the only ribonucleic acid which has been claimed to be homogeneous was prepared by relatively strong alkaline treatment of yeast (Jungner and Allgén, *Acta Chem. Scand.*, 1950, **4**, 1300). This treatment is likely to affect the internucleotide linkages and it was considered undesirable to use material prepared in this way. Thus, although determination of molecular weight is the most convenient method of determining the extent of degradation in the methylated polynucleotide, it is possible that the uncertainty arising from the polydispersity of the materials makes it insufficiently sensitive for the present purpose. It was therefore decided to seek further evidence of degradation.

There is now considerable evidence for the existence of ester linkages between positions 3′ and 5′ in adjacent nucleotides of ribonucleic acids. It follows that if the mono- and di-*O*-methylribose fractions represent corresponding structural units in the original polynucleotide as previously assumed, 2-*O*-methyl-D-ribose (and 3-*O*-methyl-D-ribose owing to

phosphoryl migration) is to be expected in the mono-*O*-methylribose fraction, but no appreciable quantity of 5-*O*-methyl-D-ribose. Examination of the mono- and di-*O*-methylribose fractions by chromatography in butanol–water and in butanol–boric acid (Barker and Smith, *loc. cit.*; Barker, Noone, Smith, and Spoors, *loc. cit.*) showed that 2-, 3-, and 5-*O*-methyl-D-ribose and 2 : 5-, 2 : 3-, and 3 : 5-di-*O*-methyl-D-ribose were present. This random distribution of the methyl groups strongly suggests that the mono-*O*-methylribose fraction arose, not only from a methylated polynucleotide chain, but also by partial methylation of fragments. It follows that the di-*O*-methylribose fraction may have arisen in a similar way and may not solely represent end groups in the polynucleotide. It seems likely, however, in view of the average molecular weights obtained by diffusion measurements, that degradation was not extensive in spite of the uncertainties mentioned above. In that case, it appeared probable that the small yield of methylated riboses obtained from the methylated product was derived mostly from small fragments rather than from the large undegraded molecules. It was, therefore, desirable to reconsider the third assumption, namely, that the sample of sugars examined was representative.

With this object in view, attempts have been made to increase the yield of methylated riboses. Although some success has been achieved, it has been impossible to obtain a recovery of more than approximately 40%. The reason for this is that in the methylated polynucleotide the phosphoryl linkages are much more stable to hydrolysis than those in the original molecule and this is believed to be because methylation of carbohydrate hydroxyl groups prevents the formation of cyclic phosphates as postulated by Brown and Todd (*loc. cit.*). It is also relevant that after hydrolysis of methylated adenylic acid, a phosphorus- and carbohydrate-containing spot was observed on the starting line in paper chromatograms and this also is believed to be due to the same effect. It is interesting that Levene and Harris (*J. Biol. Chem.*, 1932, **98**, 9) observed that a phosphoryl residue in methylated methyl riboside was resistant to hydrolysis.

From the above considerations it is concluded (*a*) that methylation cannot be used to locate a phosphoryl residue on the 2- or 3-position of ribose, (*b*) that methylation of yeast ribonucleic acid previously reported was probably incomplete, (*c*) that the methylated product contained some degraded material, and (*d*) that, owing to the stability of the phosphate ester linkages in the methylated product, the small yield of sugars obtained was not a representative sample. The last is believed to be the most important factor, since it will tend to exaggerate the effects due to (*b*) and (*c*). The authors are of the opinion that, within the limits imposed by factor (*a*) above, the technique of methylation could be applied successfully to the study of polynucleotides if a method were found for recovering all the carbohydrate residues from the methylated products. With this object in view, and also in order to provide further support for Brown and Todd's theory, a detailed study was made of the hydrolysis of methylated yeast ribonucleic acid.

The rates of mineralisation of the phosphate of yeast ribonucleic acid and methylated yeast ribonucleic acid by hot dilute acid are shown in Fig. 1. Hydrogenation (cf. Levene and LaForge, *Ber.*, 1912, **45**, 608; Levene and Jorpes, *J. Biol. Chem.* 1929, **81**, 575), and treatment with bromine (cf. Gurin and Hood, *J. Biol. Chem.*, 1941, **139**, 775; Massart and Hoste, *Biochim. Biophys. Acta*, 1947, **1**, 83) or with hydrazine (cf. Levene and Bass, *J. Biol. Chem.*, 1926—7, **71**, 167; Caputto, Leloir, Cardini, and Paladini, *ibid.*, 1950, **184**, 333) before acid hydrolysis did not materially affect the rate of mineralisation. The highest recovery was obtained when the methylated polynucleotide was subjected first to alkaline and then to acid hydrolysis. The course of the alkaline hydrolysis was therefore studied further.

In order to determine the rate of the alkaline hydrolysis of the internucleotide linkages of the methylated nucleic acid, the following procedure was adopted. At intervals during the hydrolysis, the number of equivalents of acid liberated (primary and secondary phosphate dissociations) and the number of mols. of free methanol were determined. The difference between these figures gives a value for the number of sugar–phosphate linkages broken. Aliquot parts of the hydrolysate were also neutralised and treated with prostate phosphomonoesterase and the free phosphate was determined. This gave a value for the number of monoester groups produced by the alkaline cleavage and it was found that this

equalled the number of sugar–phosphate bonds broken. It follows that in the initial rapid fission of triester residues, methanol alone is split off. Since, as can be seen from Fig. 1, little or no mineralisation of phosphate occurs, measurements of the monoester residues produced, or of the sugar–phosphate linkages broken, give the rate of fission of the internucleotide linkages in the methylated nucleic acid. A comparison of the stability of the original nucleic acid and the methylated product is shown in Fig. 2, and it is seen that fission is much slower in the methylated material. In order to confirm that this is due to the blocking of hydroxyl groups adjacent to phosphoryl residues, a similar study has been made of a specimen of yeast ribonucleic acid methylated with diazomethane, kindly supplied by Professor J. K. N. Jones. This material contained methoxyl groups attached to phosphorus, but, after hydrolysis, yielded a large quantity of ribose and only a trace of

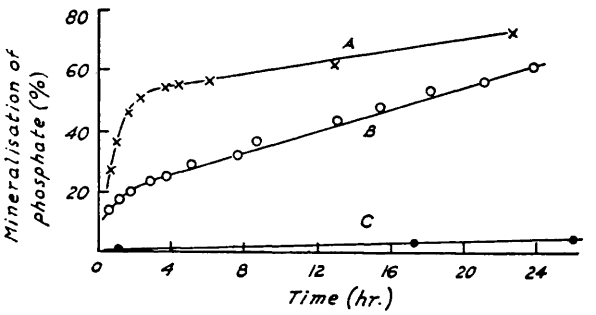

FIG. 1. *Mineralisation of phosphate.*

A, Yeast ribonucleic acid in 0·5N-sulphuric acid at 95°.
B, Methylated (MeI–Ag_2O) yeast ribonucleic acid in 0·5N-sulphuric acid at 95°.
C, Methylated nucleic acid (as for *B*) in 0·5N-sodium hydroxide at 37°.

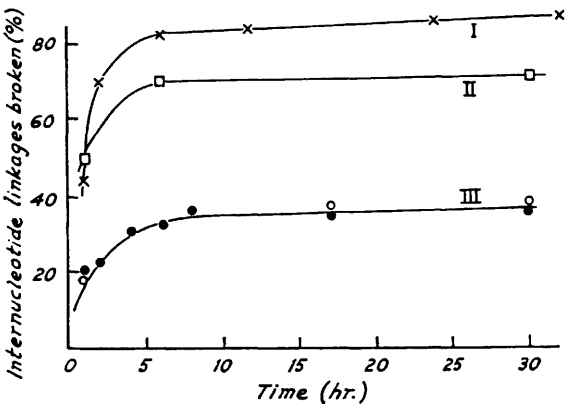

FIG. 2. *Fission of internucleotide linkages by 0·5N-sodium hydroxide.*

I, Yeast ribonucleic acid (determined by titration).
II, Yeast ribonucleic acid methylated by diazomethane (determined enzymically).
III, Yeast ribonucleic acid methylated by methyl iodide and silver oxide.

● Calc. from titration and determination of methanol.
○ determined enzymically.

methylated sugars. This indicated that, for the most part, hydroxyl groups adjacent to phosphoryl residues are not substituted. Comparison of this material with nucleic acid methylated with methyl iodide and silver oxide (see Fig. 2) indicates that the former product is more readily hydrolysed by alkali. All these considerations provide further confirmation of Brown and Todd's views.

EXPERIMENTAL

Methylation of Adenosine-3′ Phosphate.—Adenosine-3′ phosphate (0·23 g.) was methylated as previously described (Anderson *et al.*, *loc. cit.*), nine additions of silver oxide and methyl iodide being made. Removal of solvent under reduced pressure below 60° gave a pale yellow glassy product (0·26 g.) (Found: OMe, 25·0. Calc. for $C_{14}H_{21}O_4N_5$: OMe, 27·6%).

Hydrolysis of Methylated Adenosine-3′ Phosphate.—The above methylated adenylic acid in 0·5N-sodium hydroxide (45 c.c.) was kept at 37° for 17 hr. The solution was adjusted to pH 7·1 by addition of glacial acetic acid, diluted to 1750 c.c. (optical density 1·15 at 260 mμ), and run on to a column (12 × 5 cm.) of Dowex-1 resin (200—400 mesh) in the chloride form.

The column was washed with distilled water and the combined effluents (2315 c.c.; optical density 0·5 at 260 mμ) were rejected. The resin was then eluted with N-hydrochloric acid, and the fraction (732 c.c.; optical density 0·96 at 260 mμ) was collected. This solution (or, in experiments in which alkaline hydrolysis was omitted, a solution of methylated nucleotide in N-hydrochloric acid) was refluxed for 2 hr. and, after being cooled, adjusted to pH 6·9 by addition of sodium hydroxide solution. The solution was concentrated under reduced pressure to dryness, the residual solid which contained much sodium chloride was extracted repeatedly with hot methanol, and the combined extracts (300 c.c.) were again concentrated to dryness. The residue was dissolved in water (25 c.c.) and passed first through a column (10 × 1·5 cm.) of Amberlite IR-120 resin in the hydrogen form and then through a column (10 × 1·5 cm.) of Amberlite IRA-400 resin in the hydroxide form. The resulting solution was evaporated under reduced pressure to a very small volume and was applied to a sheet of Whatman No. 1 filter-paper and chromatographed with *n*-butanol–water. The paper was then dried and sprayed with saturated aqueous boric acid, care being taken to avoid wetting the original chromatographic lane. The paper was then chromatographed in *n*-butanol–boric acid in a direction at right angles to the original direction, dried, and sprayed with aniline phthalate. Besides a spot corresponding in position to D-ribose, faint spots were obtained in the region expected for dimethylriboses, but they were not strong enough to allow of identification with certainty. Discrete spots were obtained, however, which correspond in R_F value with 2-, 3-, and 5-*O*-methyl-D-ribose.

Examination of the Carbohydrate Residues of Methylated Yeast Ribonucleic Acid.—The methylated nucleic acid (Anderson *et al.*, *loc. cit.*) (0·65 g.) in 0·5N-sodium hydroxide (50 c.c.) was kept at 37° for 14 hr. N-Sulphuric acid (25 c.c.) was added and, after being diluted to 780 c.c. and made 0·5N with respect to sulphuric acid, the solution was kept at 95—100° for 1·5 hr., cooled, and passed through a column (11 × 3 cm.) of Amberlite IR-120 resin (hydrogen form) and then through a column of Amberlite IR-4B (carbonate form). The total effluent was concentrated under reduced pressure to a syrup which was found by the method of Hirst, Hough, and Jones (*J.*, 1949, 928) to contain 0·09 g. of reducing sugar expressed as ribose. Two-dimensional paper chromatography by the method described above gave spots corresponding to ribose and 2-, 3-, and 5-*O*-methyl-D-ribose. Also, three spots of approximately equal intensity were present corresponding to 2 : 3-, 2 : 5-, and 3 : 5-di-*O*-methyl-D-ribose. The mono-*O*-methylribose fraction was also examined by chromatography on a column of Dowex-1 in the borate form. Elution with 0·01M-sodium tetraborate gave three well-defined fractions, detected colorimetrically by a modification of A. H. Brown's method (*Arch. Biochem.*, 1946, **11**, 269) and shown to contain 2-, 3-, and 5-*O*-methyl-D-ribose by comparison on paper chromatograms in *n*-butanol–boric acid with synthetic material (Barker, Noone, Smith, and Spoors, *loc. cit.*).

Rate of Liberation of Acid Groups by Alkaline Hydrolysis.—Measurements were made during the hydrolysis of yeast ribonucleic acid, of yeast ribonucleic acid methylated with methyl iodide and silver oxide, and of yeast ribonucleic acid methylated with diazomethane. The following is a typical experiment. The results are shown in Fig. 1.

Aliquot parts (2 c.c.) of a solution of methylated ribonucleic acid (0·42 g.) in water (25 c.c.) were maintained at 37° with N-sodium hydroxide (2 c.c.) in stoppered tubes. At intervals, tubes were opened, N-sulphuric acid (2 c.c.) was added and, after dilution to approximately 35 c.c., the solutions were titrated electrometrically with 0·1N-sodium hydroxide. It is assumed that at pH 7·5 primary and secondary phosphoric acid dissociations have been titrated (cf. Fletcher, Gulland, and Jordan, *J.*, 1944, 33).

Liberation of Methanol during the Alkaline Hydrolysis of Methylated Ribonucleic Acids.—Methylated nucleic acid (0·28 g.) in 0·5N-sodium hydroxide (25 c.c.) was kept at 37°. At intervals, aliquot parts (2 c.c.) were withdrawn and, after being adjusted to pH 7 by the addition of 0·5N-hydrochloric acid, the solutions were diluted to 30 c.c. and methanol was determined colorimetrically (Hoffpauir and O'Connor, *Analyt. Chem.*, 1949, **21**, 420). Results are shown in Fig. 1.

Formation of Monoester Residues during the Alkaline Hydrolysis of Methylated Ribonucleic Acids.—Methylated nucleic acid (0·114 g.) in 0·5N-sodium hydroxide 10 c.c.) was kept at 37° for 47 hr. An aliquot part (5 c.c.) of the hydrolysate was brought to pH 5·1 by the addition of N-sulphuric acid and diluted to 50 c.c. Two aliquot parts (1 c.c. each) were used for the determination of total phosphorus (Allen, *Biochem. J.*, 1940, **34**, 858), and the rest of the solution was divided into two halves. To one half was added water (1 c.c.) and to the other was added a solution (1 c.c.) of prostate phosphomonoesterase (Beale, Harris, and Roe, *J.*, 1950, 1397). Both solutions were incubated at 37° and, after varying intervals of time, aliquot parts (3 c.c.) were removed and free phosphate was determined (Allen, *loc. cit.*). The final constant value

gave the number of singly esterified phosphoryl residues in the hydrolysate. The whole experiment was repeated with various times of hydrolysis with sodium hydroxide. Results are shown in Fig. 2.

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