192. *The Constitution of Yeast Ribonucleic Acid. Part XIV. The Preparation and Structures of the Pyrimidine Nucleotides.*

By G. R. Barker, (the late) J. Masson Gulland, H. Smith, and J. F. Thomas.

An improved routine laboratory preparation of cytidine-3′ phosphate and uridine-3′ phosphate from yeast ribonucleic acid is described, the latter being isolated as its crystalline *disodium* salt. It is shown that the same pyrimidine nucleotides are obtained whether the fission of the polynucleotide is carried out in alkaline or acid medium.

A survey of the older literature reveals a number of methods for preparing nucleotides from yeast ribonucleic acid (for references see Levene and Bass, " Nucleic Acids ", Chem. Cat. Co.,

New York, 1931; Gulland, *J.*, 1938, 1722; Lythgoe, *Ann. Reports*, 1944, 200), in which fission of the polynucleotide is brought about by enzymes or in acid or alkaline medium. None of the processes so far described, however, results in nucleotides in such yields as to warrant the assumption that they are the sole hydrolytic products. It was desirable, therefore, to attempt to devise methods whereby nucleotides could be obtained in higher and more reproducible yields, and this paper describes a process for the preparation of pyrimidine nucleotides which has proved technically more convenient and more consistent than those described in the literature.

After the completion of this work, a new method for the preparation of cytidylic acid and diammonium uridylate was reported by Loring, Roll, and Pierce (*J. Biol. Chem.*, 1948, **164**, 729), but the yields of cytidylic acid recorded by these authors are slightly less than we have obtained by the present procedure. The yield of diammonium uridylate obtained by the American workers is roughly comparable with that of disodium uridylate which we obtain. We find, however, that conversion of brucine uridylate into the disodium salt is much more satisfactory than the isolation of the nucleotide as its diammonium salt. Furthermore, the disodium salt is of greater use in the biological experiments for which the present investigations were undertaken. However, the yields of pyrimidine nucleotides obtained by Loring *et al.* and by us do not preclude the possibility that compounds other than 3′-phospho-pyrimidine nucleosides are formed during the fission of yeast ribonucleic acid, and this problem together with the analogous one regarding purine nucleotides will form the subject of another paper.

The separation of uridylic and cytidylic acids from a mixture of four nucleotides is a tedious and lengthy process. The use of an acid medium for the hydrolysis of the nucleic acid, however, simplifies to a large extent the subsequent operations since purine nucleotides are destroyed under these conditions. The process now described uses this means of splitting the nucleic acid and is a modification of the method of Bredereck and Richter (*Ber.*, 1938, **71**, 718), who hydrolysed yeast nucleic acid with boiling dilute sulphuric acid, and, after removal of guanine and of sulphate ions, precipitated cytidylic acid with anhydrous pyridine, in which the uridylic acid dissolved. The innovations which we have introduced into this method greatly increase the ease of manipulation and the reproducibility of the results, and overcome many of the difficulties experienced by previous workers (see Loring, Roll, and Pierce, *loc. cit.*). The most important improvements are (*a*) removal of phosphate ions together with sulphate ions, which avoids contamination of the uridylic acid with phosphoric acid and results in more consistent yields of this nucleotide; (*b*) improvement of the technique for the precipitation of cytidylic acid in such a way that it is obtained initially in a granular filtrable condition instead of as a gum; and (*c*) the isolation of uridylic acid as its crystalline *disodium* salt.

Although acid hydrolysis of pentose nucleic acids has been used for a considerable time in the preparation of pyrimidine nucleotides, no adequate comparison has been made of the materials obtained by this method and those isolated after alkaline hydrolysis. It is known that the uridylic acid obtained by acid hydrolysis of yeast nucleic acid by the method now described is uridine-3′ phosphate (Gulland and Smith, *J.*, 1947, 338). Since, however, the nature of the internucleotide linkages in the polynucleotide molecule is unknown, it cannot be predicted whether or not the same linkages are broken under acid and alkaline conditions, and it cannot, therefore, be assumed that identical nucleotides are produced in each case. In fact, there is evidence (Blumenthal and Herbert, *Trans. Faraday Soc.*, 1945, **41**, 611) that certain phosphoric esters behave differently in acid and alkaline hydrolytic fission. Yeast nucleic acid was therefore hydrolysed with cold dilute aqueous sodium hydroxide, and, after removal of the purine nucleotides by the methods of Buell and Perkins (*J. Biol. Chem.*, 1927, **72**, 21) and Steudel and Peiser (*Z. physiol. Chem.*, 1923, **127**, 262) and finally by acid hydrolysis, the pyrimidine nucleotides were separated with pyridine. In this way, cytidylic acid, disodium uridylate, and dibrucine uridylate were obtained, identical with the compounds obtained after degradation of the polynucleotide with acid. Furthermore, the same uridylic acid was obtained by deamination of cytidylic acid, obtained by acid hydrolysis, by the method of Bredereck (*Z. physiol. Chem.*, 1934, **224**, 79). It follows, therefore, that uridine-3′ phosphate and cytidine-3′ phosphate are produced from yeast ribonucleic acid, whether fission of the internucleotide linkages is carried out in acid or alkaline medium. Conversely, it may be that the unknown internucleotide linkage which is labile towards alkali is also labile towards acid, and that the stabilising effect of the pyrimidine bases on the phosphoric ester grouping at position 3′ does not operate in the case of this unknown linkage. This point can only become clear when a complete knowledge of the hydrolytic products of the polynucleotide is achieved.

Experimental.

Preparation of Cytidylic and Uridylic Acids by Acid Hydrolysis.—Yeast ribonucleic acid (Pharmco-Chemical Products Ltd.) (400 g.) was added, with stirring, to hot 2% w/v sulphuric acid (4 l.), and the solution was refluxed for 2 hours. After standing overnight, the mixture was made 3·8% w/v with respect to sulphuric acid and left for several days. Guanine sulphate was removed by filtration, and hot saturated barium hydroxide solution was added to the filtrate until the solution was alkaline to phenolphthalein. The precipitate of barium phosphate and barium sulphate was collected by centrifugation and washed 6 times by resuspension in boiling water (2 l. each) and centrifugation. The combined centrifugates were freed from barium ions quantitatively by addition of sulphuric acid and filtration, and the filtrate was concentrated under reduced pressure to a thick syrup, freedom from barium and sulphate ions being checked during the evaporation of a more concentrated solution. The thick syrup was poured into anhydrous pyridine (3 l.) which had been cooled below 0° in a freezing mixture, and the pyridine cytidylate which was formed on kneading the gum was left in the freezing mixture for 3 hours and then collected by filtration and washed with ice-cold pyridine (500 c.c.). The combined filtrate and washings were evaporated under reduced pressure to a thick syrup, and the pyridine treatment repeated. The filtrate and washings were evaporated as before, and a portion of the syrup tested with pyridine. Usually the precipitation of pyridine cytidylate was complete at this stage, but the above procedure was repeated if necessary. The combined granular precipitates constituted the cytidylic acid fraction, the filtrates from which were concentrated under reduced pressure to a syrup (uridylic fraction).

Cytidylic Fraction.—The pyridine cytidylate was dissolved in water (2 l.) and evaporated to a syrup under reduced pressure to remove uncombined pyridine. The residue was dissolved in water (1·5 l.), and lead cytidylate precipitated by addition of 25% solution of lead acetate (about 400 c.c.), the pH of the solution being kept at 7·2 by addition of ammonia solution (*d* 0·88) (about 25 c.c.) until precipitation was complete. The lead salt was collected by centrifugation and washed 4 times by stirring vigorously with water (2 l.) followed by centrifugation. Passage of hydrogen sulphide for 5 hours through a stirred suspension of the lead salt in water (3 l.) formed a solution of the free acid. The mixture was heated to 80°, passage of hydrogen sulphide being continued, and the coagulated lead sulphide was removed by filtration (charcoal), washed with hot water, and resuspended in water (3 l.). The mixture was saturated with hydrogen sulphide, and, with hydrogen sulphide passing through the solution, the temperature was raised to 80° for 1 hour. The lead sulphide was again removed by filtration, and washed with hot water. The combined filtrates and washings were aerated to remove excess of hydrogen sulphide and concentrated under reduced pressure to 400—500 c.c., and the solution was boiled with charcoal and filtered. To the boiling filtrate, boiling absolute industrial alcohol (about 600 c.c.) was added until an incipient turbidity was produced. On seeding, crystals of cytidylic acid separated immediately in needles, which, after being kept in the refrigerator for several days, were collected (23 g.) and dried in a vacuum over phosphoric oxide. The material was recrystallised from boiling water (25 parts) by addition of boiling absolute industrial alcohol (about 25 parts) (Found in the anhydrous material: C, 33·4; H, 4·4; N, 12·7; P, 9·4. Calc. for $C_9H_{14}O_8N_3P$: C, 33·4; H, 4·3; N, 13·0; P, 9·6%). In a 1 mm. tube in a bath at 220°, the temperature rise being 5° per minute, the needles melted at 231—233° (vigorous decomp.). In water the material dried as for analysis showed $[\alpha]_D^{25°}$ +36·7° (*c*, 2·2). In 2% aqueous sodium hydroxide the material dried as for analysis had $[\alpha]_D^{25°}$ +22·7 (*c*, 1·1).

Uridylic Fraction.—The pyridine uridylate was converted into the free nucleotide as described for cytidylic acid. The filtrates and washings from the lead sulphide were freed from hydrogen sulphide by aeration, and evaporated under reduced pressure to 200 c.c. A small quantity was titrated with sodium hydroxide solution, using phenolphthalein, and brucine (5% in excess of that theoretically required to form dibrucine uridylate) dissolved in alcohol (100 c.c.) was added to the bulk of the solution. On stirring and seeding, brucine uridylate crystallised, and, after being kept overnight in the refrigerator, was collected by filtration. After being recrystallised twice from 33% alcohol (80 parts) in the presence of charcoal it had m. p. 185—195° under the standard conditions given by Gulland and Smith (*loc. cit.*) (yield 63 g.) [Found in anhydrous material: C, 59·1; H, 6·1; N, 7·6; P, 2·8. Calc. for $C_9H_{13}O_9N_2P,(C_{23}H_{26}O_4N_2)_2$: C, 59·3; H, 5·9; N, 7·6; P, 2·8%]. Sufficient N-sodium hydroxide solution was added to a well-stirred suspension of the brucine salt in boiling water (600 c.c.) in order to convert all of it into the disodium salt. After the mixture had been allowed to cool and left overnight, the bulk of the brucine was filtered off and washed with water, and the remainder removed from the filtrate and washings by extraction with chloroform. The solution of *disodium uridylate* was evaporated to 50 c.c., filtered through charcoal, and cooled in ice. Absolute industrial alcohol was added very slowly with mechanical stirring until a turbidity was first formed. The solution was then seeded, and the addition of alcohol was continued periodically, with stirring, until complete crystallisation had occurred (about 500 c.c.). After the mixture had been kept in the refrigerator overnight, the needles (18 g.) were collected at the pump. They were recrystallised by dissolving them in hot water (25 c.c.), filtering the solution in the presence of charcoal, cooling in ice, seeding, and adding absolute industrial alcohol very slowly (about 250 c.c.) as before (Found in the anhydrous material: C, 29·8; H, 3·2; N, 7·5; P, 8·4. $C_9H_{11}O_9N_2PNa_2$ requires C, 29·4; H, 3·2; N, 7·6; P, 8·4%). Loss in weight on drying, 19·3; the tetrahydrate requires 19·6%. The anhydrous material had in water $[\alpha]_D^{25°}$ +19·8° (*c*, 2·25). The sodium salt was dissolved in water and the rate of dephosphorylation by N/10-sulphuric acid at 100° was carried out under the same conditions as those described by Gulland and Smith (*loc. cit.*), with the following results (0·48 mg. of P per 10 c.c. of solution):

Hours	1	3	5	10	22	24
Dephosphorylation, %	8	17	28	45	71	77

Cytidylic acid (11 g.) was deaminated as described by Bredereck (*loc. cit.*), and after purification through the brucine salt, sodium uridylate (4·4 g.) was obtained as described above. The anhydrous material had in water $[\alpha]_D^{24°}$ +21·4° (*c*, 1·0).

Preparation of Uridylic and Cytidylic Acids by Alkaline Hydrolysis.—Yeast ribonucleic acid (Pharmco-Chemical Products Ltd.) (200 g.) was hydrolysed by 5 l. of 1% sodium hydroxide solution at room temperature for 3 days. Guanylic acid was removed as its ammonium salt by the method of Buell and Perkins (*loc. cit.*), and adenylic acid crystallised directly as described by Steudel and Peiser (*loc. cit.*). The mother liquors from the crystallisations of the adenylic acid were combined, and after hydrolysis at 100° in 2% w/v sulphuric acid (2 l.) for 2 hours, cytidylic and uridylic acids were isolated as described above for the acid hydrolysate. The following were obtained :

Cytidylic acid (4 g.), which, when placed in a 1 mm. tube in a bath at 220° the temperature rise being 5° per minute, melted at 231—233° (vigorous decomp.); when mixed with a sample of cytidylic acid prepared by acid hydrolysis it had m. p. 231—233° under the same conditions; in water, the material dried as for analysis had $[\alpha]_D^{25°}$ +37·0° (*c*, 2·2); in 2% sodium hydroxide solution it had $[\alpha]_D^{25°}$ +21·8° (*c*, 1·1).

Dibrucine uridylate (6 g.), m. p. and mixed m. p. with sample of brucine uridylate prepared by acid hydrolysis of the nucleic acid 185—195° under the standard conditions given by Gulland and Smith (*loc. cit.*).

Disodium uridylate (1·6 g.), which (anhydrous material) had in water $[\alpha]_D^{26°}$ +20·0° (*c*, 2·64); it was dissolved in water, and the rate of dephosphorylation by N/10-sulphuric acid at 100° was carried out under the same conditions as those described by Gulland and Smith (*loc. cit.*), with the following results (0·48 mg. of P per 10 c.c. of solution) :

Hours	1	3	5	10	22	24
Dephosphorylation, %	6	19	27	42	68	73

It is a pleasure to record our gratitude to the British Empire Cancer Campaign for financial assistance. Our thanks are due to Mr. J. E. Still for microanalyses.

THE UNIVERSITY, MANCHESTER, 13.
THE UNIVERSITY, NOTTINGHAM.

[*Received, September 14th*, 1948.]