392. *The Isolation of Uridine from Yeast Ribosenucleic Acid.*

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An improved method has been found for the isolation of uridine from yeast ribosenucleic acid. It is now possible to separate and identify the two purine and two pyrimidine nucleosides from small samples of the nucleic acid.

The chemical investigation of small amounts of pentosenucleic acids which may be derived from different plant and animal sources is difficult, and the identity of these acids has frequently been assumed on the evidence of unspecific colour reactions for the identification of the pentose or of qualitative tests for the nitrogenous bases. Where larger amounts are available, it is usually

possible to isolate the individual nucleotides or nucleosides for comparison with those obtained by similar methods from yeast ribosenucleic acid. Thus it has been established that adenylic, guanylic, cytidylic, and uridylic acids may be obtained from wheat-embryo nucleic (" triticonucleic ") acid (Calvery and Remsen, *J. Biol. Chem.*, 1927, **73**, 593) which are identical with those obtained from yeast. Similarly two purine and two pyrimidine nucleosides have been obtained from chick-embryo nucleic acid (Calvery, *ibid.*, 1928, **77**, 489), and more recently Schwerdt and Loring (*ibid.*, 1947, **167**, 593) were able to isolate very small quantities of diammonium uridylate, cytidylic acid, and brucine guanylate from tobacco-mosaic virus pentosenucleic acid.

As a preliminary to the study of the nucleic acids of normal and tumour tissue an investigation has been made of the preparation of the purine and pyrimidine nucleosides of yeast ribosenucleic acid.

Gulland and Hobday (*J.*, 1940, 746), using a modification of the Levene method of ammonia hydrolysis (Levene and Bass, " Nucleic Acids ", 1931, Reinhold Publishing Corp., p. 162; Levene and Jacobs, *Ber.*, 1909, **42**, 2471, 2476; 1910, **43**, 3154), prepared 6 g. of uridine from 100 g. of B.D.H. yeast nucleic acid and in this preparation all the cytidine present was deaminated to uridine.

Bredereck, Martini, and Richter (*Ber.*, 1941, **74**, 694) claimed to have obtained 2—3 g. of uridine and 4 g. of cytidine (no melting points or analytical data are given) from 100 g. of nucleic acid by either enzymatic or aqueous pyridine hydrolysis. Since both methods gave identical yields it is reasonable to assume that losses were due, not to incomplete hydrolysis, but to poor separative methods. Gulland and Smith (*J.*, 1947, 338) modified Bredereck's method and, incorporating the cytidine deamination procedure, obtained yields of 17·5—22·5 g. of uridine from 360 g. of nucleic acid. These authors were unable, after several attempts, to prepare cytidine by Bredereck's method.

Despite the large quantities of nucleic acid used, demonstration of all four nucleosides in the same hydrolysis sample has not readily been forthcoming.

In the method to be described, guanosine, adenosine, and uridine can be obtained in good yield from the pyridine hydrolysate of quite small (20 g.) quantities of yeast nucleic acid, and smaller quantities of cytidine (as cytidine sulphate) have been isolated at the same time. From the removal of adenosine to the crystallisation of uridine the Gulland–Bredereck procedure involves seventeen stages, whereas uridine and cytidine can now be separated in one operation.

The amino-group in cytidine has pK 4·22, and the nucleoside is adsorbed from aqueous solution by suitably-prepared cation-exchange resins. When a mixture of cytidine and uridine is percolated through a column of " Zeo-Karb 215 ", cytidine is retained by the resin and uridine alone appears in the effluent. Washing with water removes the uridine quantitatively, and the cytidine may subsequently be recovered by treatment of the resin with aqueous pyridine.

For convenience in handling the column a simple method of determining uridine and cytidine has been used. In aqueous solution both pyrimidine nucleosides react quantitatively at room temperature with free bromine with the formation of 5-bromouridine and 5-bromocytidine respectively. Free bromine is obtained by the normal bromide–bromate method, and reacts : Uridine + Br_2 = 5-bromouridine + HBr.

The operation is conducted in reagent bottles fitted with ground-glass stoppers, and the excess of bromine is determined iodometrically. Guanosine, adenosine, and free pentose (in this case, arabinose) do not interfere under the conditions of the determination. Uracil (and, presumably, cytosine) does interfere, and no selectivity is claimed for this method. It is useful, however, in the analysis of solutions of known composition where other methods of nucleoside determination are cumbersome.

This method of separating the pyrimidine ribosides is equally applicable to the separation of the pyrimidine deoxypentosides derived from deoxypentosenucleic acids, and an investigation of these acids has been started.

The successful recovery of pyrimidine ribosides from small quantities of yeast nucleic acid indicates that it will now be possible to obtain chemical data for the identification of the hydrolysis products of those nucleic acids which are not available on a centigramme or even decigramme scale.

Experimental.

Preparation of a Column of Cation-exchange Resin.—The exchange column consisted of a vertical glass tube 50 cm. in length and 3·5 cm. in diameter, having a side-arm 10 cm. from the top and fitted at the bottom with a sintered glass disc (to support the resin) and a tap. Enough " Zeo-Karb 215 " (The Permutit Co., Ltd.) to fill the column to a height of 27 cm. was soaked for 24 hours in sufficient

hydrochloric acid (2 parts acid, *d* 1·18, to 1 part water) to cover it. The column was then washed backwards with just sufficient head of distilled water to produce a gentle turbulence. The washing water flowed out through the side-arm. This method of washing serves the double purpose of grading the bed and effecting the removal of very fine debris which would otherwise clog the column. The resin in this condition actively removes cations from solution. It may be exhausted with sodium chloride solution (hydrochloric acid appearing in the effluent) and regenerated by treatment with 2N-hydrochloric acid. Usually an exchanger is ready for use after two or three exhaustion-regeneration cycles. After each regeneration the column is first back-washed with distilled water as described above and the washing completed by percolation in the normal direction until the effluent is free from chloride ion.

Preparation of Specimens of Guanosine, Adenosine, Cytidine, Uridine.—By the method of Bredereck *et al.* (*loc. cit.*) comparable yields were obtained of guanosine [colourless plates, m. p. 240° (sinters 235°), from water], and of adenosine (colourless needles, m. p. 228°, from water). Adenosine picrate, yellow plates, had m. p. 182°.

The pyrimidine nucleoside-containing residues were used to prepare uridine (in poor yield) by the method of Gulland and Hobday (*loc. cit.*). Uridine crystallised as colourless needles, m. p. 165—166°, from dilute alcohol; 5-bromouridine, needles, had m. p. 181—183°.

Cytidine was prepared by pyridine hydrolysis of cytidylic acid (Gulland and Smith—private communication). 6 G. of cytidylic acid gave 3 g. of cytidine as colourless needles, m. p. 230—233°, from dilute alcohol. Cytidine sulphate also crystallised in needles, m. p. 224—225° (decomp.), from dilute alcohol containing a drop of dilute sulphuric acid.

Levene records m. p. of 233° for cytidine sulphate, but the lower figure is accepted by Howard, Lythgoe, and Todd (*J.*, 1947, 1052) for the sulphate of synthetic cytidine.

Volumetric Determination of Uridine and Cytidine.—Hydrochloric acid (10 c.c.; N) and potassium bromide solution (5 c.c.; 10%) were added to the nucleoside solution (10 c.c.) (containing 1—10 mg.) or water (10 c.c.) (for determination of the blank) in a small reagent bottle fitted with a ground-glass stopper. The solution was titrated with 0·01N-potassium bromate until excess of bromine was present. It was usually an advantage to determine roughly the amount of bromate required and to use a 50% excess in further determinations. The same excess could then be used in the blank estimation. The bottle was then stoppered and allowed to stand at room temperature for 20 minutes. Potassium iodide solution (5 c.c.; 10%) was added, and the liberated iodine titrated immediately with 0·01N-sodium thiosulphate, using a starch indicator.

1 c.c. of 0·01N $KBrO_3$ = 1·216 mg. of uridine.

The accuracy of the determinations is indicated in the table.

Uridine present (mg.).	Cytidine present (mg.).	Uridine found (mg.).	Cytidine found (mg.).	Uridine present (mg.).	Cytidine present (mg.).	Uridine found (mg.).	Cytidine found (mg.).
0·984	—	0·984	—	4·920	—	4·860	—
1·968	—	1·930	—	—	1·999	—	1·960
3·836	—	3·750	—	—	3·998	—	3·995
4·920	—	4·840	—	—	4·998	—	4·970
4·920	—	5·020	—	0·492 +	5·050	5·650	
				2·461 +	2·525	5·090	

The last two results show the determination of total nucleoside in a mixture of cytidine and uridine. In comparable quantities adenosine and arabinose took up no bromine.

Behaviour of Individual Nucleosides on the Exchanger Column.—In these experiments a smaller column of resin was employed of length 14 cm. and diameter 1·7 cm. This had one-tenth the cation capacity of the larger column described above.

Adenosine. 50 C.c. of solution containing 250 mg. of adenosine were percolated through the column, which was then washed with an equal volume of water. The combined effluents were evaporated to dryness. No adenosine or solid material was present. The column was then stripped with 2% pyridine (100 c.c.), and the effluent concentrated under reduced pressure at 30—40°. Addition of a saturated solution of picric acid in water gave a quantitative yield of a picrate, as yellow platelets, m. p. 182° (undepressed by admixture with adenosine picrate). Elution of the column with 20% pyridine instead of 2%, however, produced considerable heat, the adenosine was hydrolysed *in situ*, and adenine picrate, m. p. 278—280° (decomp.), was recovered.

Cytidine. In similar experiments it was found that cytidine was retained completely by the resin and could be liberated with 2% pyridine.

Uridine. A solution of 250 mg. of uridine in 50 c.c. of water was percolated through the column in the usual way. The combined effluents contained (by volumetric determination, in which 10 c.c. of water washings of the column before use gave a negligible blank) 246 mg. of uridine thus: (*a*) 12 c.c. (= "dead space"), nil; (*b*) 50 c.c., 181 mg.; (*c*) 50 c.c., 65 mg.; (*d*) 50 c.c., nil.

Cytidine–uridine mixture. The solution contained 99·7 mg. of uridine and 37·0 mg. of cytidine in 100 c.c. 200 C.c. of initial effluent contained, by analysis, 95·5 mg. of uridine. The 2% pyridine effluent contained 26·0 mg. of cytidine.

The Separation of Uridine and Cytidine from the Pyridine Hydrolysate of Yeast Ribosenucleic Acid.—The hydrolysis of 20 g. of yeast nucleic acid (Pharmaco-Chemical Products Co.) with 50% pyridine and the separation of the guanosine and adenosine were carried out substantially by the method of Bredereck, Martini, and Richter (*loc. cit.*), and similar yields were obtained. The filtrate from the precipitation of the adenosine was concentrated under reduced pressure with repeated addition of water until all the alcohol had been removed. Residual purine nucleosides were hydrolysed with 2% sulphuric acid, and the liberated purines precipitated with hot silver sulphate solution. Excess of silver was removed as silver sulphide, and the solution was freed from SO_4^{--} quantitatively by addition of barium carbonate and diluted to 1 l. Percolation through the larger exchange column and washing with water gave 2 l. of effluent,

which, on concentration under reduced pressure, yielded 3·1 g. of brown crystalline material. Recrystallisation from dilute alcohol (charcoal) gave 2·4 g. of colourless needles, m. p. 165—166°, undepressed by admixture with uridine prepared by the Gulland–Bredereck procedure. $[\alpha]_D^{22°}$ + 10·0° ± 2° (*c*, 1·6245 in water) (Found, in material dried at 80°/0·1 mm.: C, 43·8; H, 5·0. Calc. for $C_9H_{12}O_6N_2$: C, 44·2; H, 5·0%).

The column was then stripped in the usual way with 2% pyridine (1·6 l.), and washed with water (650 c.c.). The combined solutions were concentrated under reduced pressure to 10 c.c., 0·7 c.c. of concentrated sulphuric acid was added, and cytidine sulphate precipitated by addition of alcohol. Recrystallisation from dilute alcohol containing a drop of dilute sulphuric acid gave 150 mg. of colourless needles, m. p. 224—225° (decomp.) undepressed by admixture with cytidine sulphate prepared from cytidylic acid. $[\alpha]_D^{21°}$ + 37·0° ± 2° (*c*, 1·498 in 1% aqueous sulphuric acid) [Found, in material dried at 110°/0·1 mm.: C, 37·3; H, 4·7. Calc. for $(C_9H_{13}O_5N_3)_2,H_2SO_4$: C, 37·0; H, 4·8%].

This low yield of cytidine is most probably due to destruction of the nucleoside by the acid treatment designed to hydrolyse the residual purine nucleosides.

In a further separation of a similarly-derived mixture, 1·95 g. of uridine and 140 mg. of cytidine sulphate were obtained. The amounts of uridine made available by this process are thus considerably in excess of those normally obtained by previous methods.

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