427. *The Isolation of Uridine and Cytidine from Yeast Ribonucleic Acid.*

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An improved method for the separation and isolation of uridine and cytidine from yeast ribonucleic acid is described. It has been shown that hydrolysis of yeast ribonucleic acid, using aqueous pyridine, is incomplete under the conditions used by Bredereck, Richter, and Martini (*Ber.*, 1941, **74**, 694). Appreciable deamination of cytidine occurs during the process.

The usual technique employed for the preparation of nucleosides from pentose nucleic acids is that due to Bredereck *et al.* (*Ber*, 1941, **74**, 694). Although this method affords satisfactory yields of guanosine and adenosine, and, by a suitable modification, a fair quantity of uridine also (Gulland and Smith, *J.*, 1947, 338), the isolation of cytidine has proved difficult. Thus Gulland and Smith (*J.*, 1948, 1527) were compelled to resort to the hydrolysis of cytidylic acid, and Harris and Thomas (*J.*, 1948, 1936) obtained extremely small yields of cytidine after hydrolysis of yeast ribonucleic acid with aqueous pyridine. Loring and Ploeser (*J. Biol. Chem.*, 1949, **178**, 439) have recently described a method for preparing the pyrimidine nucleosides by acid hydrolysis of yeast ribonucleic acid. Unfortunately, however, the conditions of hydrolysis employed caused considerable deamination of the cytidine.

It therefore seemed desirable to investigate more fully the degradative method of Bredereck *et al.* Some reference to this problem is made by Markham and Smith (*Nature*, 1949, **163**, 250) who state that " the hydrolysis of ribonucleic acid by pyridine–water is far from complete, and the hydrolysate contains at least seven different substances." This agrees with the observations reported herein, since hydrolysis of yeast ribonucleic acid with 50% aqueous pyridine for 103 hours removed only 57% of the total phosphorus as inorganic phosphate. Furthermore, examination of the hydrolysate by paper chromatography revealed the presence of considerable quantities of nucleotides.

A method, similar in principle to that described by Harris and Thomas (*Nature*, 1948, **161**, 931; *J.*, 1948, 1936), was employed for the separation of synthetic mixtures of cytidine and uridine (Elmore, *Nature*, 1948, **161**, 931). This has now been adapted for the isolation of these nucleosides from hydrolysates of yeast ribonucleic acid. Yields of uridine were of the same order as those reported by Harris and Thomas (*locc. cit.*). Although yields of cytidine were considerably higher than obtained by these authors, they must still be regarded as low, especially since Vischer and Chargaff (*J. Biol. Chem.*, 1948, **176**, 715) report that cytosine is three times as abundant as uracil in yeast ribonucleic acid. In agreement with Loring and Ploeser (*loc. cit.*) and contrary to the suggestion of Harris and Thomas (*J.*, 1948, 1936), it was found that destruction of cytidine did not occur to any marked extent under the conditions of acid hydrolysis which are normally employed to remove traces of purine nucleosides. The small yields of cytidine may be accounted for, however, since it has been found that deamination of the cytosine nucleoside or nucleotide occurs during the initial hydrolysis with aqueous pyridine. Cytidylic acid, when subjected to these conditions, afforded an appreciable quantity of uridine, which was detected by paper-chromatographic examination of the hydrolysate. The extent of deamination may be judged from the fact that cytidine sulphate was obtained from cytidylic acid in 68% yield.

It has been found advantageous to delay hydrolysis of residual purine nucleosides until after the separation and isolation of uridine. This modification in the procedure circumvents the difficulty of crystallization of uridine frequently encountered, which is attributed to the presence of a small amount of gummy material formed during the acid hydrolysis.

Experimental.

Preparation of Uridine.—A sample of uridine, m. p. 163—165°, was prepared from yeast ribonucleic acid by a combination of the methods of Bredereck *et al.* (*loc. cit.*) and Gulland and Hobday (*J.*, 1940, 746).

Preparation of Cytidine from Cytidylic Acid.—The method employed was that due to Gulland and Smith (*J.*, 1948, 1527). The yield was 68%, and the m. p. 228—230° (decomp.).

Preparation of " Zeo-Karb 215."—The resin was alternately crushed in an iron mortar and sieved until it passed a 30-mesh but was retained by a 40-mesh size. Before use the resin was freed from soluble polymers by subjecting it four times to the following cycle of operations: treatment with 2N-hydrochloric acid, treatment with water until the washings were neutral, treatment with sodium hydroxide, and treatment with water until the washings were neutral. The sodium hydroxide used was 0·5N. in the first two, and 0·1N. in the last two, cycles. Finally, the resin was treated with 2N-hydrochloric acid,

washed with water, and dried at room temperature. Treatment with acid or alkali was carried out in a downward direction in a column. Washing with water was carried out in the reverse direction.

Behaviour of Cytidine on "Zeo-Karb 215."—A solution (0·05M.) of cytidine (0·61 g.) in water (50 c.c.) was passed down a column (4·2 × 1·2 cm.) of "Zeo-Karb 215" (2 g.) at a rate of 1·5 c.c./min., and the percolate collected in fractions. Kjeldahl analyses of the latter demonstrated that cytidine was quantitatively retained. By determination of the "break-through point," the capacity of the resin for cytidine under these conditions was found to be 1·25 milliequivs./g. The column was eluted with 0·1N-ammonia (100 c.c.), the eluate evaporated under reduced pressure to remove ammonia (Nessler test), and the solution analysed by the Kjeldahl technique. Recovery was 81%.

Behaviour of Uridine on "Zeo-Karb 215."—In a similar experiment it was shown that uridine was not retained by a column of "Zeo-Karb 215."

Separation of a Synthetic Mixture of Cytidine and Uridine.—A solution (0·05M. with respect to each nucleoside) of cytidine (0·61 g.) and uridine (0·61 g.) in water (50 c.c.) was passed down a column (5·6 × 1·2 cm.) of "Zeo-Karb 215" (3 g.) at a rate of 0·7 c.c./min. The column was washed with water (100 c.c.), and the combined washings and filtrate were evaporated to dryness, yielding crystalline uridine (0·608 g., 99·8%), m. p. 162—165° (Found: N, 11·5. Calc. for $C_9H_{12}O_6N_2$: N, 11·5%).

The column was eluted with 0·1N-ammonia (100 c.c.), and the eluate evaporated to dryness, affording crystalline cytidine (0·433 g., 71%), m. p. 208—211° (Found: N, 17·0. Calc. for $C_9H_{13}O_5N_3$: N, 17·3%).

Preparation of Cytidine and Uridine from the Pyridine Hydrolysate of Yeast Ribonucleic Acid.—Yeast ribonucleic acid (100 g.) was hydrolysed with 50% aqueous pyridine by the method of Bredereck *et al.* (*loc. cit.*), and the guanosine and adenosine were removed as described by these authors. Yields of guanosine were 10—14 g. and of adenosine 6—11 g.

The mother-liquors were diluted to 1500 c.c. and passed down a column (28·5 × 3·0 cm.) of "Zeo-Karb 215" (90 g.) at a rate of 1·2 c.c./min. The column was washed with water (700 c.c.), and the combined percolate and washings were evaporated under reduced pressure to a syrup. After evaporation once with alcohol, the syrup solidified to a brown mass of crude uridine which was crystallized from 95% alcohol (yield, 16·65 g.). Recrystallized from 95% alcohol, it (11·95 g.) had m. p. 163·5—166° alone or on admixture with a sample of uridine prepared by the Gulland–Bredereck procedure, $[\alpha]_D^{16}$ +9·6° (*c*, 2·0 in water) (Found: C, 44·7; H, 4·7; N, 11·5. Calc. for $C_9H_{12}O_6N_2$: C, 44·3; H, 4·9; N, 11·5%).

The column was eluted with 0·1N-ammonia (5 l.) and the eluate evaporated under reduced pressure to 200 c.c. Sulphuric acid (2·2 c.c.) was added, the solution was heated under reflux for 1·5 hours and cooled, and purines were precipitated by the addition of hot silver sulphate solution. Silver purines were filtered off and washed twice with cold water, and the combined washings and filtrate evaporated under reduced pressure to 500 c.c. Silver ions were removed by hydrogen sulphide and, after aeration of the solution, barium hydroxide was added to pH 2·2—2·4. Barium sulphate was filtered off and washed with hot water, and the combined washings and filtrate were evaporated to 60 c.c. Boiling alcohol was added to the hot solution until crystallization commenced. The cytidine sulphate obtained (3·65 g.) had m. p. 224—225° (decomp.), $[\alpha]_D^{17}$ +37·5° (*c*, 1·5 in 1% aqueous sulphuric acid) [Found: C, 37·2; H, 5·13; N, 14·5. Calc. for $(C_9H_{13}O_5N_3)_2,H_2SO_4$: C, 37·0; H, 4·8; N, 14·4%]. A second preparation gave 3·96 g. of cytidine sulphate.

Cytidine sulphate (2 g.) was converted by the method of Gulland and Smith (*J.*, 1948, 1527) into the free nucleoside (1·30 g.), m. p. 212—215° (undepressed on admixture with cytidine prepared from cytidylic acid), $[\alpha]_D^{16}$ +34·2° (*c*, 2·0 in water) (Found: C, 44·7; H, 5·3; N, 17·2. Calc. for $C_9H_{13}O_5N_3$: C, 44·4; H, 5·35; N, 17·3%).

Behaviour of Cytidine in Boiling 2% Sulphuric Acid.—Cytidine (0·3 g.) was heated under reflux in 2% w/v sulphuric acid (20 c.c.) for 2 hours. The solution was cooled and tested with Nessler's solution. A yellow colour was noticeable but no precipitate appeared, showing that deamination of cytidine had not occurred to any marked extent under these conditions.

Behaviour of Cytidylic Acid in Boiling 50% Aqueous Pyridine.—Cytidylic acid (1 g.) was heated under reflux in 50% aqueous pyridine (50 c.c.) for 100 hours (extent of hydrolysis, 82%). The solution was chromatographed on acid-washed Whatman No. 1 filter paper (Hanes and Isherwood, *Nature*, 1949, **164**, 1107), using *tert.*-butanol–pyridine–water (60 : 15 : 25). Uridine, cytidine, and uridylic and cytidylic acids were used as markers. The chromatogram was examined by photographing it in ultra-violet light on Ilford Reflex Document Paper No. 50 (Markham and Smith, *loc. cit.*; *Biochem. J.*, 1949, **45**, 294). Pronounced spots corresponding to uridine (R_F 0·67) and cytidine (R_F 0·51) and a fainter one corresponding to cytidylic acid (R_F 0·24) were observed.

Chromatographic Examination of the Aqueous Pyridine Hydrolysate of Yeast Ribonucleic Acid.—Yeast ribonucleic acid (1 g.) was heated under reflux in 50% aqueous pyridine (60 c.c.) for 103 hours (extent of hydrolysis, 57%). The solution was chromatographed as above. Guanosine (R_F 0·51), adenosine (R_F 0·59), cytidine (R_F 0·51), uridine (R_F 0·67), guanylic acid (R_F 0·26), yeast adenylic acid (two spots; R_F values, 0·24 and 0·30), cytidylic acid (R_F 0·24), and uridylic acid (R_F 0·36) were used as markers. The hydrolysate gave five spots with R_F values 0·24, 0·34, 0·52, 0·59, and 0·66.

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