chromatography. The mononucleotide product is almost exclusively uridylic and cytidylic acids in approximately equal amounts. The small quantity of adenylic and guanylic acid appearing as mononucleotides seems to arise from higher molecular weight compounds during the analytical separation on the ion-exchange resin. Several discrete fractions, which were bound more strongly to the anion exchanger than are mononucleotides, were analyzed and each was found to have a different polynucleotide composition. These latter fractions may be artifacts resulting from the analytical procedure or polynucleotide subgroups arising from the action of nuclease on nucleic acid or degradation products of such polynucleotides.

OAK RIDGE, TENNESSEE RECEIVED OCTOBER 24, 1949

[CONTRIBUTION FROM THE BIOLOGY DIVISION OF THE OAK RIDGE NATIONAL LABORATORY]

The Preparation of Uridylic and Cytidylic Acids from Yeast Ribonucleic Acid by an Ion-exchange Method[1]

BY W. E. COHN AND C. E. CARTER

The separation of uridylic and cytidylic acids from mixtures of hydrolytic products of yeast ribonucleic acid by the methods described by Levene[2] is a tedious process with low yields. Recently Loring, Roll and Pierce[3] reported a new method involving fractionation of the phosphotungstate salts of the nucleotides for the preparation of uridylic and cytidylic acids which offers improvement of yield and simplification over previous procedures. A modification of the method of Brederek and Richter,[4] in which uridylic and cytidylic acids are separated in anhydrous pyridine recently described by Barker, Gulland, Smith and Thomas,[5] also greatly improves the yield and ease of preparation of these compounds. Although the ion-exchange preparation of pyrimidine nucleotides offers no advantage over the latter methods in terms of purity of the final product and only a slight improvement of yield, the comparative ease with which unequivocal separation of the pyrimidine nucleotides from each other and from remaining hydrolytic products is achieved with this technique contributes significantly to the usefulness of the method in nucleotide chemistry.

The procedure employed for the preparation of uridylic and cytidylic acids entails the following steps: (1) hydrolysis in 1 *N* sulfuric acid of yeast ribonucleic acid to adenine, guanine, uridylic acid, cytidylic acid, ribose, phosphate and traces of unidentified hydrolytic products, (2) removal of sulfuric acid and most of the guanine and inorganic phosphate by neutralizing the hydrolysate with barium hydroxide, (3) separation of remaining adenine, guanine and the two pyrimidine nucleotides by acid elution from a strong base anion exchanger, (4) crystallization or precipitation of each pyrimidine nucleotide from the portion of the column effluent in which it is recovered. The analytical methods of anion exchange separation of nucleotides described by Cohn,[6] have been adapted to the preparative procedures described in this paper.

Experimental

One hundred grams of Schwarz yeast nucleic acid was added to 700 ml. of 1 *N* sulfuric acid and the mixture heated with stirring over a burner to 95°, during which time the nucleic acid dissolved. The solution was then transferred to a steam-bath, kept for two hours at 95° and cooled to room temperature. (Under these conditions, there was no significant liberation of inorganic phosphate after ninety minutes.) The hydrolysate was adjusted to *p*H 2.0 with barium hydroxide, and barium sulfate was removed by centrifugation without loss of inorganic or organic phosphate.

Preparation I.—To the supernatant remaining after the removal of barium sulfate, more saturated barium hydroxide solution was added to a *p*H of 7.5 and the precipitate was removed by centrifugation. This precipitate was extracted with hot water and an aliquot submitted to paper chromatography[7]; it was thus shown to contain mainly adenine and guanine (and inorganic phosphate), and about four grams of pyrimidine nucleotide. This extract was discarded and only the supernatant remaining after the removal of the precipitate at *p*H 7.5 was put on the anion exchanger.

The anion exchanger, Amberlite IRA-400 of bed size 13 cm. × 33 sq. cm., was converted to the formate form by washing with sodium formate and formic acid, then with distilled water to an effluent *p*H of 3.0. The supernatant (1980 ml.) remaining after the removal of the barium precipitate at *p*H 7.5, was put through the column, followed by water. The effluent was examined in the spectrophotometer, as previously described.[6] An aliquot of the first 2500 ml. effluent from the column (of *p*H 5.1) was chromatographed[7] and shown to contain adenine, a trace of guanine and no uridylic or cytidylic acid.

The column was then washed with 1 *N* formic acid and the effluent analyzed spectrophotometrically[6] and by paper chromatography[7]; the following were recovered: (1) 0–750 ml., 0.25 g. of cytidylic acid; (2) 750–1700 ml., 8.0 g. of cytidylic acid; (3) 1700–2150 ml., 1.7 g. of cytidylic acid, 0.3 g. of uridylic acid. Fractions (1) and (3) were discarded, and (2) was saved for crystallization of cytidylic acid.

The normality of the formic acid solution was then raised to 2.4; the effluent analyzed[6,7] as follows: (1) 0–4 liters, 0.75 g. of uridylic plus cytidylic acid; (2) 4–11 liters, 11.7 g. of uridylic acid; (3) 11–12 liters, 0.5 g. of uridylic

(1) Operated under Contract Number W-7405-Eng-26 for the Atomic Energy Commission, Oak Ridge, Tennessee.

(2) P. A. Levene and C. W. Bass, "Nucleic Acids," American Chemical Society Monograph Series, Reinhold Publ. Corp., New York, N. Y., 1931.

(3) H. S. Loring, *et al.*, *J. Biol. Chem.*, **174**, 729 (1948).

(4) J. Brederek and R. Richter, *Ber.*, **71**, 718 (1938).

(5) G. R. Barker, *et al.*, *J. Chem. Soc.*, 904 (1949).

(6) W. E. Cohn, THIS JOURNAL, **71**, 2275 (1949); **72**, 1471 (1950).

(7) C. E. Carter, *ibid.*, **72**, 1466 (1950).

acid. Fractions (1) and (3) were discarded and Fraction (2) set aside for the preparation of diammonium uridylate.

Preparation II.—After bringing the *p*H of the nucleic acid hydrolysate to 7.5 with barium hydroxide, ammonium hydroxide was added to *p*H 8.5, and the precipitate removed by centrifugation. The precipitate was washed with three 200-ml. portions of hot water, the washes combined and added to the supernatant. Employing this procedure, no pyrimidine nucleotide was lost (dissolving the washed precipitate in acid and chromatographing it revealed only guanine) but less inorganic phosphate was removed as the insoluble barium salt than in Preparation I where the precipitate was discarded without washing.

The anion exchanger (Amberlite IRA-400, the same column as used in Preparation I) was converted to the chloride form by washing with 4 *M* hydrochloric acid, followed by water until the *p*H of the effluent rose to 3.0. The supernatant and washings from the barium precipitation stage were put through the column at a *p*H of 8.5. Under these conditions, the column approached saturation because of the additional phosphate and adenine obtained from the combined washes of the precipitate; analysis of the break-through and subsequent water washes showed: A(1) 0–500 ml. of adenine and inorganic phosphate; (2) 500–1700 ml. of adenine, cytidylic, uridylic acid, 1220 mg. of inorganic P; (3) 1700–6700 ml. of adenine. Fraction A(1) and A(3) were discarded and Fraction A(2) was set aside for recycling.

The column was then treated with 0.1 *N* hydrochloric acid and the effluent fractions analyzed: B(1) 0–800 ml. no purine or pyrimidine; (2) 800–1200 ml., 10.0 g. of cytidylic acid, no inorganic phosphate; (3) 1200–1420 ml., 3.0 g. of cytidylic acid, 1.0 g. of uridylic acid, 260 mg. of inorganic phosphate; (4) 1420–3320 ml., 2.35 g. of cytidylic acid, 17.30 g. of uridylic acid, 260 mg. of inorganic phosphate. Fraction B(2), which by ultraviolet spectrum and chromatographic analysis was shown to contain only cytidylic acid, was set aside for crystallization of this compound. Fractions B(1) and B(3) were discarded and Fraction B(4), containing predominantly uridylic acid, was saved for recycling.

The column was then washed with 4 *N* hydrochloric acid and water for cleaning and conversion to the chloride form. Fraction A(2), 1200 ml. in volume, was recycled on the column and yielded 1 g. of cytidylic acid and 1.2 g. uridylic acid, free of adenine and inorganic phosphate. The uridylic acid was combined with Fraction B(4) which, after dilution to 5 liters and adjustment of the *p*H to 10.5, was similarly fractionated on the column to yield: C(1) 0–300 ml., 0.1 g. of uridylic acid, 8 mg. of inorganic P, (2) 300–530 ml., 1.5 g. of uridylic acid, 10 mg. of inorganic P; (3) 530–2100 ml., 14.6 g. of uridylic acid, no inorganic P; (4) 2100–3800 ml., 0.4 g. of uridylic acid. Fraction C(3) was saved for the preparation of uridylic acid; all others were discarded.

Crystallization of Cytidylic Acid.—In Preparations I and II, the procedure was essentially the same. The solution containing the cytidylic acid was reduced to a volume of about 150 ml. under vacuum distillation and decolorized with norit. Cytidylic acid was precipitated by the addition of an equal volume of ethanol. The solution was then kept at −10° overnight; the crystals were filtered, washed with alcohol and ether, and dried in a vacuum desiccator over calcium chloride; yield, 6.1 g. (Prepn. I), 7.0 g. (Prepn. II); melting point, 235° with decomposition (Prepn. I and II); optical rotation in 0.5% aqueous solution, $\alpha^{20}D$ +37.9° (Prepn. I), +33.2° (Prepn. II). *Anal.* Calcd.: N, 13.0; P, 9.6. Found: N, 12.8; P, 9.72; (Prepn. I) N, 12.76; P, 9.68 (Prepn. II). To the supernatant remaining from the first crystallizations, 200 ml. of absolute ethanol was added and another 1.5 g. (0.8 g. from Prepn. II) of cytidylic acid melting at 232° were recovered.

When cytidylic acid recovered from Preparations I and II was recrystallized from 50% ethanol, the $\alpha^{20}D$ of the samples rose to 39.1 and 38.9°, respectively. Levene[2] gives $\alpha^{20}D$ 38.5° and Loring, *et al.*,[3] reported values as high as 43.0°. Both Preparations I and II were analyzed by ion exchange[6] and paper chromatography[7] and shown to contain only cytidylic acid. The molar extinction coefficients of the recrystallized preparations were 12,300 (I) and 12,120 (II) at 278 mμ; Ploeser and Loring reported 12,700.[8]

Preparation of Diammonium Uridylate.—The formic acid eluate from Preparation I, which contained about 10.0 g. of uridylic acid in 6 liters of solution, was concentrated to a sirup by vacuum distillation; water was added and the process repeated several times to remove formic acid. The sirup was then taken up in a small amount of water, decolorized with norit and again concentrated to a sirup *in vacuo*. An excess of ammonium hydroxide was added and methanol slowly introduced in an attempt to effect crystallization. A gummy precipitate resulted, so the ammonia and methanol were removed by vacuum distillation and the process repeated. After eight such attempts a crystalline product was obtained which was allowed to stand at 4° for two days, filtered (on a day when the humidity was low) and the precipitate immediately washed with absolute ethanol and ether. It was then placed in a vacuum desiccator over calcium chloride for two weeks before analysis; yield, 4.9 g. (a satisfactory product could not be recovered from the mother liquor); melting point 185° with decomposition. *Anal.* Calcd.: N, 15.7; P, 8.57. Found: N, 15.6, P, 8.5. The molar extinction coefficient in 0.01 *N* hydrochloric acid at 262 mμ was 10,030; Ploeser and Loring[8] reported 9980. The optical rotation of diammonium uridylate reported by Levene[2] and Loring, *et al.*,[3] is $\alpha^{20}D$ 21° for a 2% solution in water. For the preparation described above, $\alpha^{20}D$ 18.6° in 2.0% aqueous solution. Recrystallization from methanol–water raised the constant to 20.2°. The crystalline product was analyzed by ion exchange and paper chromatography and only one component, uridylic acid, was found.

Preparation of Barium Uridylate.—The difficulties encountered in the crystallization of diammonium uridylate make the isolation of uridylic acid as the barium salt or as the sodium salt reported by Barker, *et al.*,[5] a simpler and more economical procedure.

Fraction C(3) from Preparation II, containing 14.6 g. of uridylic acid in 1575 ml. of 0.1 *N* hydrochloric acid, was concentrated by vacuum distillation to a volume of 200 ml., one liter of water added and the concentration repeated. A standard solution of barium hydroxide, carbonate free, was added to the uridylic acid solution at the glass electrode with rapid stirring until a *p*H of 8.8 was reached. The addition was performed rapidly to minimize formation of barium carbonate. The precipitate was collected by centrifugation at 4° for thirty minutes. A small amount of precipitate formed in the supernatant after standing overnight at 4°; the two precipitates were combined, washed with alcohol and dried in a vacuum desiccator over calcium chloride. The yield was 17.8 g. of dry barium salt which contained 5.95% phosphorus. (In theory barium uridylate should contain 6.7% phosphorus. A small amount of barium chloride and carbonate probably accounts for the discrepancy.) On spectrophotometric analysis the barium salt was found to be 52% uridylic acid, but since this salt was to serve only for the preparation of free uridylic acid, no attempt was made to purify it to the theoretical uridylic acid content of 70%.

Preparation of Uridylic Acid.—For conversion to uridylic acid, 0.580 g. of the barium salt was dissolved in 25 ml. of 0.4 *N* hydrochloric acid and the solution allowed to flow through a column of the cation exchanger Dowex-50 in the H^+ form, bed size 3 cm. × 8 cm.[2]. This procedure is based on the finding[9] that uridylic acid is not retained on a cation exchanger in acid solution, while barium is strongly bound.

The content of uridylic acid in the starting solution was found to be 300 mg. by spectrophotometric analyses and of that amount, 291 mg. (or 95%) was recovered in 42.4 ml. of the column effluent (from starting solution plus

(8) J. McT. Ploeser and H. S. Loring, *J. Biol. Chem.*, **178**, 431 (1949).

(9) W. E. Cohn, *Science*, **109**, 377 (1949).

water wash). Aliquots of this solution assayed as follows: *Anal.* Calcd.: N, 8.66; P, 9.6. Found: N, 8.72; P, 9.52. The optical rotation of a 0.5% solution in 0.24 *N* hydrochloric acid, $\alpha^{20}D$ +8.55°.

Summary

The preparation of uridylic and cytidylic acids from an acid hydrolysate of yeast ribonucleic acid employing the anion exchanger Amberlite IRA-400 and elution with formic and hydrochloric acids is described. From 100 g. of nucleic acid, 4.9 g. of crystalline diammonium uridylate were obtained in a preparation involving formic acid elution and conversion of uridylic acid to the diammonium salt. In another preparation, employing a hydrochloric acid elution, 17.8 g. of a barium uridylate fraction were obtained which, by spectrophotometric assay, contained 52% (9.25 g.) uridylic acid. A method for conversion of barium uridylate to free uridylic acid employing a cation exchange technique is described. Cytidylic acid was readily crystallized from fractions eluted from the column in either dilute formic or hydrochloric acid. The yield was 7.5 g. and 6.9 g. from 100 g., respectively, of yeast ribonucleic acid.

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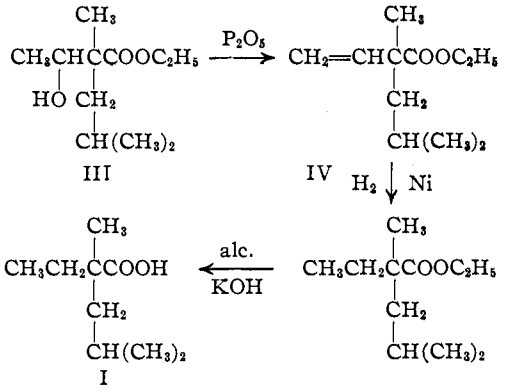
[CONTRIBUTION FROM HAVEMEYER LABORATORY, COLUMBIA UNIVERSITY]

The Synthesis and Resolution of Methylethylisobutylacetic Acid

BY W. VON E. DOERING AND KENNETH B. WIBERG

In connection with the resolution and reactions of (+)methylethylisobutylcarbinol,[1] the corresponding acid, methylethylisobutylacetic acid (I) has been synthesized and resolved.

Attempts to prepare I by carbonation of the Grignard reagent from methylethylisobutylcarbinyl chloride by the conventional method produced I in impractically low yield.[2,3] The absence of rearrangement in the dehydration of β-hydroxy-α,α-disubstituted butyric acids[4,5] encouraged the synthesis of I from methylisobutylacetoacetic ester (II). Catalytic hydrogenation of II,[6] slow with ordinary Raney nickel, but conveniently rapid with "W-5" Raney nickel,[7]



produced the requisite β-hydroxyester (III) which was dehydrated with phosphorus pentoxide to methylisobutylvinylacetic ester (IV). Hydrogenation of IV followed by hydrolysis gave the desired acid I. The structure of IV was supported by ozonolysis to an aldehyde, oxidation and hydrolysis of which gave the known methylisobutylmalonic acid (V).[8] It is of interest that the oxidation of the acid derived from IV (VI) with alkaline potassium permanganate gave mainly methylisobutylacetic acid and a small amount of methylisobutyl ketone, but only a trace of V despite the inertness of the latter to permanganate.[9]

The resolution of I, effected by the fractional crystallization of the brucine salt, appears to be the first reported resolution of a trialkyl acetic acid in which the carboxylic acid group is attached to the asymmetric center.[10] The (+) acid obtained by this resolution had an infrared absorption curve which appeared to be identical with that of the *dl*-acid (see Fig. 1).[11] In addition, methylisobutylvinylacetic acid (VI) was partially resolved by means of the (+)α-phenylethylamine salt. Hydrogenation of (+)VI gave (+)I.

(1) Doering and Zeiss, THIS JOURNAL, **70**, 3966 (1948), **72**, 147 (1950), and unpublished results.

(2) This result parallels that of Schuerch and Huntress, *ibid.*, **70**, 2824 (1948).

(3) The recent modification of Lester and Proffitt [*ibid.*, **71**, 1877 (1949)] in which methylethyl-*n*-propylacetic acid is prepared by the carbonation of the Grignard reagent under pressure with shaking gives good yields.

(4) Shive, Crouch and Lochte, *ibid.*, **63**, 2979 (1941).

(5) Courtot, *Bull. soc. chim.*, [3] **35**, 118 (1906).

(6) *Cf.* Adkins, Connor and Cramer, THIS JOURNAL, **52**, 5192 (1930); Covert and Adkins, *ibid.*, **54**, 4116 (1932).

(7) Adkins and Billica, *ibid.*, **70**, 695 (1948).

(8) Burrows and Bentley, *J. Chem. Soc.*, **67**, 510 (1895).

(9) Courtot, ref. 5, reports that dimethylvinylacetic acid is oxidized by alkaline permanganate to dimethylmalonic acid in 72% yield. The anomalous oxidation of IV acid is being investigated.

(10) Conant and Carlson, THIS JOURNAL, **54**, 4048 (1932), reported the resolution of methyl-*n*-butylphenylacetic acid. In this connection it might be noted that camphene-1-carboxylic acid [Houben and Pfankuch, *Ber.*, **59**, 956 (1926)]; umbellularic acid [Rydon, *J. Chem. Soc.*, 829 (1936)]; and camphoric acid [Debierne, *Compt. rend.*, **128**, 1112 (1899)] have also been resolved. However, each of these contains two asymmetric centers.

(11) The infrared absorption curves were kindly obtained by Mr. A. P. Wolf of these laboratories using a Perkin-Elmer Model 12-B infrared spectrometer modified for automatic ratio recording. [Abraham Savitzky, Doctoral Dissertation, Columbia University, 1949.]