Nucleotides. Part XXVIII. A Synthesis of Uridine-5′ Triphosphate (UTP).*

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Uridine-5′ triphosphate has been synthesised by condensing 2′ : 3′-di-*O*-acetyl- or 2′ : 3′-*O*-*iso*propylidene-uridine-5′ benzyl phosphorochloridate with a salt of tribenzyl pyrophosphate, and then removing the benzyl and other protecting groups. The synthetic product is identical with uridine triphosphate (UTP) (III) isolated from natural sources. The synthesis of uridine-5′ pyrophosphate (UDP) (Kenner, Todd, and Weymouth, *J.*, 1952, 3675) has been improved.

AFTER the isolation of adenosine triphosphate from muscle extracts (Lohmann, *Naturwiss.*, 1929, **17**, 624; Fiske and Subbarow, *Science*, 1929, **70**, 381), various other coenzymes were recognised (*e.g.*, coenzyme I, FAD) as derivatives of adenosine, but until recently no substances of a similar nature were known which were derived from any other natural ribonucleoside. In 1950, however, Caputto, Leloir, Cardini, and Paladini (*J. Biol. Chem.*, 1950, **184**, 333) described the isolation of uridine-diphosphate-glucose (UDPG) which acts as coenzyme in the system which converts galactose into glucose. Since then related compounds have been reported, including uridine-diphosphate-glucuronic acid (Dutton and Storey, *Proc. Biochem. Soc.*, 1953, **53**, xxxvii; Smith and Mills, *Biochem. Biophys. Acta*, 1954, in the press), uridine-diphosphate-*N*-acetylglucosamine (Cabib, Leloir, and Cardini, *J. Biol. Chem.*, 1953, **203**, 1055), and even more complex uridine diphosphate derivatives (Park, *ibid.*, 1952, **194**, 877, 885). Kornberg (*Symp. Phosphorus Metabolism*, 1951, **1**, 392) suggested that uridine-5′ triphosphate (UTP) (III) might be an intermediate in the enzymic synthesis of such uridine derivatives and was indeed able to demonstrate that uridine triphosphate could be prepared enzymically from uridine diphosphate (uridine-5′ pyrophosphate).

As part of our general programme on nucleoside polyphosphate synthesis we prepared uridine-5′ triphosphate in the latter part of 1952. Our immediate object was then to demonstrate the validity of a synthetic method and no serious attempt was made to increase the purity of the final product above about 50%. The material in this state of purity was, however, adequate for biochemical studies, and it was used in investigations which demonstrated the production of urdine-5′ triphosphate by the uridyl transferase-catalysed pyrophosphorolysis of UDPG (Munch-Petersen, Kalckar, Cutolo, and Smith, *Nature*, 1953, **172**, 1036) and from UDPG and uridine-diphosphate-*N*-acetylglucosamine in presence of a rat-liver nuclear fraction (Smith, Munch-Petersen, and Mills, *ibid.*, p. 1038). The growing biochemical interest in uridine triphosphate encouraged us to continue work on its synthesis. Bergkvist and Deutsch (*Acta Chem. Scand.*, 1953, **7**, 1307) meanwhile

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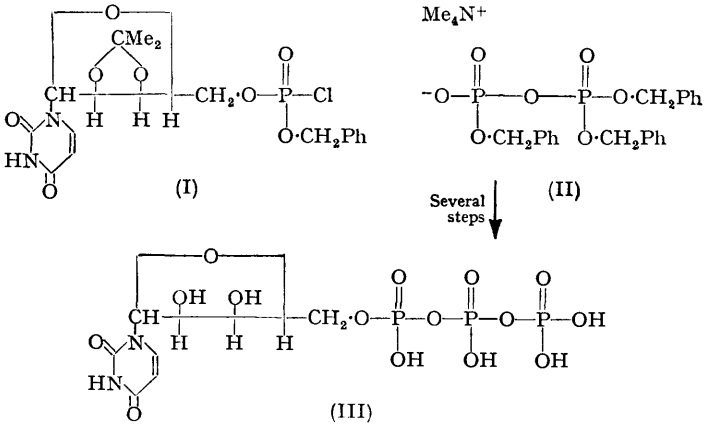
detected uridine triphosphate and guanosine triphosphate in anion-exchange chromatogram fractions of yeast and muscle extracts, and Lipton, Morell, Frieden, and Bock (*J. Amer. Chem. Soc.*, 1953, **75**, 5449) isolated uridine triphosphate in substance from yeast using anion-exchange methods.

The methods employed in this laboratory for the synthesis of adenosine-5′ triphosphate (Part II, Baddiley, Michelson, and Todd, *J.*, 1949, 582; Part IV, Michelson and Todd, *ibid.*, p. 2487) are not readily applicable to the corresponding uridine derivative (cf. Part XV, Anand, Clark, Hall, and Todd, *J.*, 1952, 3665). It was therefore decided to extend the method used by Kenner, Todd, and Weymouth (Part XVII, *ibid.*, p. 3675) for uridine-5′ pyrophosphate (UDP) and to synthesise uridine-5′ triphosphate by condensing a suitable protected derivative of uridine-5′ benzyl phosphorochloridate with a salt of tribenzyl pyrophosphate and subsequently removing the benzyl and other protecting groups. The major difficulty in polyphosphate syntheses of this nature lies in the extreme lability of the fully esterified products obtained after the initial condensation, and the consequent need for a method by which at least partial debenzylation to more stable intermediates can be effected smoothly and rapidly. In the earlier syntheses of uridine-5′ pyrophosphate (Parts XV and XVII, *locc. cit.*) partial debenzylation was effected by lithium chloride, but in the present work we used phenols as debenzylating agents based on as yet unpublished experiments by Dr. A. S. Curry and Mr. J. Mather in this laboratory. In a preliminary experiment, use of this method for the partial debenzylation of tribenzyl 2′ : 3′-*O*-*iso*propylideneuridine-5′ pyrophosphate (Part XVII, *loc. cit.*), followed by hydrogenolysis of remaining benzyl groups and removal of the *iso*propylidene residue with acid, gave considerably improved yields of uridine-5′ pyrophosphate.



Since no information was then available about the stability of uridine triphosphate, 2′ : 3′-di-*O*-acetyluridine was used as starting material, for it could be assumed that mild deacetylation with catalytic amounts of alkali-metal alkoxides would not cause extensive destruction of the triphosphate residue. Accordingly 2′ : 3′-di-*O*-acetyluridine-5′ benzyl phosphite was treated with *N*-chlorosuccinimide and the resulting phosphorochloridate was brought into reaction with triethylammonium tribenzyl pyrophosphate. The resinous product, presumably mainly 2′ : 3′-di-*O*-acetyluridine-5′ tetrabenzyl triphosphate, was partially debenzylated with hot phenol, and the isolated product was deacetylated with sodium methoxide in methanol. The remaining benzyl groups were removed by hydrogenolysis and the crude uridine-5′ triphosphate isolated as a sodium salt. Paper-chromatographic analysis indicated that the main component of the crude product showing ultra-violet absorption (*ca.* 50% of the total absorption at 260 mμ) was uridine-5′ triphosphate, the remainder being uridine-5′ pyrophosphate and uridine-5′ phosphate, doubtless arising by decomposition of initially formed triphosphate. Independent assays of this material by paper chromatography (Smith and Mills, *loc. cit.*) and by biological methods (Berg and Joklik, *Nature*, 1953, **172**, 1008) confirmed our own findings.

From the behaviour of this crude material it was clear that uridine-5′ triphosphate was more stable to acids than we had feared and so for subsequent syntheses we used the more accessible 2′ : 3′-*O*-*iso*propylideneuridine as starting material. In the final method 2′ : 3′-*O*-*iso*propylideneuridine-5′ benzyl phosphorochloridate (I) was condensed with the crystalline tetramethylammonium tribenzyl pyrophosphate (II), the product at once partially debenzylated with *m*-cresol containing a little hydrogen chloride, and the removal of benzyl groups then completed by hydrogenolysis. Subsequent removal of the *iso*propylidene residue and precipitation as a lithium salt yielded a material containing *ca.* 37% of uridine-5′ triphosphate (III), the impurities being the 5′-pyrophosphate and 5′-phosphate and inorganic phosphates. Further purification by charcoal chromatography or, much more conveniently, by anion-exchange chromatography (Dowex-2) gave pure uridine-5′ triphosphate which we isolated as the barium salt. Through the courtesy of Dr. S. A. Morell we were able to identify the synthetic product with natural uridine triphosphate by direct comparison with a specimen of the sodium salt isolated in the Pabst Laboratories (Milwaukee) from yeast. The synthetic method described is straightforward in use and on the scale employed has given yields of *ca.* 29% based on uridine.

Experimental

Uridine-5′ Barium Pyrophosphate.—Crude tribenzyl 2′ : 3′-*O*-*iso*propylidene uridine-5′ pyrophosphate (prepared from 1·94 g. of benzyl 2′ : 3′-*O*-*iso*propylidene uridine-5′ phosphite; Kenner, Todd, and Weymouth, *J.*, 1952, 3679) and dry phenol (12 g.) were heated at 50° for 17 min. Water (140 c.c.) and ethanol (60 c.c.) were added and the resulting homogeneous solution (approx. N/20) was hydrogenated overnight at atmospheric pressure with a mixture of palladous oxide and 10% palladium–charcoal. The catalysts were removed by filtration, the filtrate was adjusted to pH 4·5 with sodium hydroxide, and the phenol removed in ether (5 × 100 c.c.). The aqueous solution was evaporated to 10 c.c. under reduced pressure (final pH 3·5), barium bromide (1·7 g.) was added, and the precipitate (90 mg.) immediately formed was filtered off and discarded. Ethanol (28 c.c.) was added to the filtrate (18 c.c.), and the precipitate was centrifuged off, washed with acetone and ether, and dried, giving a white powder (1·40 g.). Paper chromatography in *iso*propanol–1% ammonium sulphate solution (3 : 2) showed that this product contained, in addition to uridine-5′ pyrophosphate, only small amounts of uridine-5′ phosphate and 2′ : 3′-*O*-*iso*propylidene uridine-5′ pyrophosphate.

The above barium salt was dissolved as completely as possible in 0·1N-hydrochloric acid (10 c.c.), the mixture set aside at room temperature for 5 hr. and then filtered, and the filtrate neutralised to pH 6 with aqueous barium hydroxide. Ethanol (30 c.c.) was added, and the precipitate collected, washed with acetone and ether and dried, giving a white powder (0·99 g., 34% based on 2′ : 3′-*O*-*iso*propylideneuridine). Paper chromatography as before showed the product to be virtually pure uridine-5′ pyrophosphate (Found: C, 15·8; H, 3·1; N, 3·6. $C_9H_{11}O_{12}N_2P_2Ba_{1\frac{1}{2}},5H_2O$ requires C, 15·5; H, 3·0; N, 4·0%).

2′ : 3′-*Di*-O-*acetyluridine.*—Triphenylmethyl chloride (11·4 g.) was added to a solution of uridine (10 g.) in pyridine (100 c.c.). The mixture was kept at room temperature for 2 days and then at 100° for 3 hr. Acetic anhydride (80 c.c.) was added to the cooled solution which, after a further 20 hr., was poured into vigorously stirred ice-water (800 c.c.). The flocculent precipitate was collected and dried (26·4 g.). A mixture of this material and 80% acetic acid (90 c.c.) was boiled under reflux for 7 min. before concentration to small bulk under reduced pressure. Addition of water (200 c.c.) gave a suspension, which was extracted with benzene (3 × 70 c.c.) and then filtered. The aqueous solution was concentrated to 100 c.c. and extracted with ethyl acetate (6 × 30 c.c.), which yielded on evaporation a pale yellow resin (6·4 g.), which slowly crystallised. It was recrystallised by dissolution in the minimum quantity of acetone and addition of ether (2 vols.), followed by sufficient pentane to produce slight turbidity. This *diacetate* (6·05 g., 45%) was used for further work, but the m. p. could be raised by repeated recrystallisation from 133—134° to 142—143° (Found, in material dried at 70°: C, 47·7; H, 5·2; N, 8·6. $C_{13}H_{16}O_8N_2$ requires C, 47·6; H, 4·9; N, 8·5%).

2′ : 3′-*Di*-O-*acetyluridine*-5′ *Benzyl Phosphite.*—Application of the method described by Kenner, Todd, and Weymouth (*loc. cit.*) for the *iso*propylidene compound to 2′ : 3′-di-*O*-acetyluridine (0·90 g.) gave a pale yellow resin, which was purified by pouring a solution in benzene (10 c.c.) into *cyclo*hexane (200 c.c.). The precipitated gum (1·12 g., 85%) appeared to be homogeneous on paper chromatography (R_F 0·78 in *n*-butanol–water) and was converted by

successive chlorination with *N*-chlorosuccinimide, hydrolysis with aqueous sodium hydrogen carbonate, deacetylation with sodium methoxide, and hydrogenolysis into material indistinguishable by paper chromatography in *iso*propanol–1% ammonium sulphate (3 : 2) from uridine-5′ phosphate.

Tetramethylammonium Tribenzyl Pyrophosphate.—Silver tribenzyl pyrophosphate was prepared by the method of Baddiley, Clark, Michalski, and Todd (*J.*, 1949, 818) and recrystallised by adding 5 vols. of water to a filtered solution in the minimum quantity of boiling acetone (Found, in the hygroscopic material dried at 60° : C, 45·0; H, 4·0. Calc. for $C_{21}H_{21}O_7P_2Ag$: C, 45·4; H, 3·8%). To a solution of silver tribenzyl pyrophosphate (5·55 g.) in acetone (200 c.c.) was added one of tetramethylammonium iodide (2·01 g.) in water (100 c.c.). The mixture was refluxed for 5 min., then filtered through " Hyflo Supercel," the filtrate was evaporated to dryness, and the last traces of moisture were removed by repeated evaporation with benzene. The waxy residue on crystallisation from benzene–*cyclo*hexane gave fine, colourless, hygroscopic needles of *tetramethylammonium tribenzyl pyrophosphate* (4·8 g.), m. p. 74—75° (Found, in material dried at room temperature : N, 2·85; P, 11·7. $C_{25}H_{33}O_7NP_2$ requires N, 2·7; P, 11·9%).

Triethylammonium tribenzyl pyrophosphate was similarly prepared by dissolving silver tribenzyl pyrophosphate in chloroform, adding the theoretical quantity of solid triethylammonium chloride, filtering off the precipitated silver chloride, and evaporating *in vacuo*. The triethylammonium salt obtained as a gum was dried before use by repeated evaporation with benzene.

Uridine-5′ Triphosphate.—(*a*) A mixture of benzyl 2′ : 3′-di-*O*-acetyluridine-5′ phosphite (1·95 g.) and *N*-chlorosuccinimide (0·54 g.) in methyl cyanide (12 c.c.) was kept at room temperature for 2 hr. A solution of triethylammonium tribenzyl pyrophosphate (prepared from 2·47 g. of silver tribenzyl pyrophosphate) in methyl cyanide (20 c.c.) was slowly added, followed by a few drops of triethylamine, and the solution was set aside for 2 hr. at room temperature. The solvent was evaporated *in vacuo*, the residue dissolved in molten phenol (25 g.), and the solution heated at 60° for 15 min. Water (100 c.c.) was then added and the solution extracted with ether (200 c.c., then 3 × 100 c.c.). The aqueous solution was neutralised to pH 7·5 with N-sodium hydroxide (1·5 c.c.), evaporated to 70 c.c., and freeze-dried, affording a resin (3·26 g.) which was dissolved in methanol (20 c.c.). A solution of phenolphthalein in methanol (2 drops) was added, followed by sodium methoxide in methanol until a permanent pink colour was obtained. A gel was formed after 5 min., and after 30 min. solid carbon dioxide was added, followed by acetone (20 c.c.). The precipitate was centrifuged off and dissolved in water, and the solution brought to pH 4 with hydrochloric acid and hydrogenated at atmospheric pressure with a mixed palladous oxide–palladised charcoal catalyst. The hydrogen uptake (53 c.c.) was complete in 1·5 hr. The catalyst was filtered off, and the filtrate neutralised to pH 7 with sodium hydroxide, evaporated to 5 c.c., and poured into acetone (40 c.c.). The precipitated oil was centrifuged off and dried under reduced pressure, to give a stable frothy glass (867 mg.). Paper chromatography in *iso*propanol–1% ammonium sulphate solution (3 : 2) showed the nucleotide content of the final product to be approximately : uridine-5′ triphosphate 40—50, pyrophosphate 25—30, and phosphate 25—30%. The ultraviolet absorption at 260 mμ showed that nucleotides accounted for about 72% of the weight of the crude material. The yield of uridine-5′ triphosphate obtained was therefore approx. 12%.

(*b*) A mixture of benzyl 2′ : 3′-*O*-*iso*propylideneuridine-5′ phosphite (3·6 g.) and *N*-chlorosuccinimide (1·18 g.) in benzene (10 c.c.) and methyl cyanide (15 c.c.) was gently stirred at room temperature for 2 hr. A solution of tetramethylammonium tribenzyl pyrophosphate (4·62 g.) in methyl cyanide (20 c.c.) was added and the stirring continued for a further 2½ hr. The mixture was filtered and the precipitate of tetramethylammonium chloride (0·4 g.) was washed with benzene (5 c.c.). The combined filtrate and washings were evaporated *in vacuo*, the residue was dissolved in dry *m*-cresol, and the solution was made up to 100 c.c. with *m*-cresol. To 50 c.c. of the above solution a solution of hydrogen chloride in dry *m*-cresol (5·0 c.c. of 0·5N) was added and the mixture set aside for 2 hr., during which a gelatinous mass separated. Ethanol (55 c.c.) and water (50 c.c.) were added and the resulting homogeneous solution was hydrogenated at atmospheric pressure with a mixed palladium oxide–palladised-charcoal (5%) catalyst. The hydrogenation was interrupted after 3 hr. (uptake 158 c.c.), and the catalyst was filtered off and washed with water (5 c.c.). The combined filtrate and washings were diluted with water (150 c.c.), extracted with ether (4 × 100 c.c.), further diluted with water (to 350 c.c.), and set aside at 5° for 12 hr. The solution was neutralised to pH 7·0 with N-lithium hydroxide (19·3 c.c.), evaporated to 20 c.c. *in vacuo*, and filtered into acetone (220 c.c.). The precipitate

was centrifuged off, washed with acetone (2 × 220 c.c.), then with ether (100 c.c.), and dried over phosphoric oxide *in vacuo*, affording a fine white powder (2·1 g.). The remaining 50 c.c. of the cresol solution of tetrabenzyl uridine-5′ triphosphate, similarly treated, also gave a fine white powder (2·0 g.). Paper chromatography in *iso*propanol–1% ammonium sulphate showed that the material contained uridine-5′ triphosphate (5%) and uridine-5′ diphosphate (5%), together with much material having a negative reaction with a periodate spray (Buchanan, Dekker, and Long, *J.*, 1950, 3162). The total product (4·1 g.) was therefore dissolved in water (160 c.c.), divided into 4 portions of 40 c.c., and each portion in turn was passed through a column (4 × 4 cm.) of cation-exchange resin (Zeocarb 315, H^+ form); the column was washed with water (40 c.c.), and the combined effluents were hydrogenated overnight as above. Filtrate from the catalyst was neutralised with N-lithium hydroxide (9 c.c.). The solutions so obtained were combined, evaporated to small bulk (25 c.c.), and poured into acetone (220 c.c.), and the precipitate was centrifuged off, washed with acetone (2 × 200 c.c.) and with ether (200 c.c.), and dried over phosphoric oxide, yielding a white powder (3·4 g.). Paper chromatography in *iso*propanol–1% ammonium sulphate solution (3 : 2), followed by elution of the spots with 0·01N-hydrochloric acid and determination of the amounts of individual nucleotides by ultra-violet absorption measurements at 260 mμ, gave uridine-5′ triphosphate 41, pyrophosphate 29·4, and phosphate 29·6%. Determination of the ultra-violet absorption of a dilute solution of the product showed that these nucleotides accounted for 74% of the weight of the crude material. The actual yield of uridine-5′ tetralithium triphosphate was thus 1·26 g. (29% from uridine).

Charcoal Chromatography of Uridine-5′ Triphosphate.—A solution of the mixed sodium salts described in (*a*) above (1·17 g., containing approx. 30% of inorganic phosphate and pyrophosphate) in water (50 c.c.) was applied to a column (8 cm. × 4·5 cm. diam.) of charcoal ("Karbak;" 40 g.; previously purified by being washed with 2N-hydrochloric acid, by continuous extraction of the solid with water, ethanol, *iso*propanol and again water, and by drying and activation at 350° for 72 hr.), supported on "Hyflo Supercel" (10 g.). The column was eluted with, successively, water, 15% and 30% aqueous ethanol, and ethanol; the eluant was collected in an automatic fraction collector (9-c.c. fractions; flow rate 1 c.c./1 min.), and the progress of elution followed by paper chromatography in *iso*propanol–1% ammonium sulphate (3 : 2). With water elution, fractions 11—20 contained only inorganic pyrophosphate (eluant pH 2); fractions 21—50 contained only pyrophosphate and uridine triphosphate (pH 2, rising to 5); fractions 51—135 contained only uridine triphosphate; fractions 136—230 contained only traces of ultra-violet absorbing material. Subsequent elution with aqueous ethanol gave solutions containing uridine mono-, di-, and tri-phosphates. Fractions 60—90 from the water elution described above were pooled, neutralised to pH 6 with N-sodium hydroxide, evaporated to small bulk, and finally freeze-dried. The residue was dissolved in water (0·8 c.c.), the solution filtered into ethanol (4 c.c.), and the precipitate centrifuged off. This was examined by paper chromatography (see below). To a solution of the precipitate in 0·05N-hydrochloric acid (4 c.c.) was added barium acetate (0·5 c.c. of a saturated solution), and the precipitate was centrifuged off, washed with water (2 × 1·5 c.c.), ethanol (2 × 1·5 c.c.), and ether (2 × 1·5 c.c.), and dried *in vacuo* over phosphoric oxide, to give *uridine-5′ barium triphosphate* (27 mg.) as a fine white powder [Found, in material dried over phosphoric oxide at 0·1 mm.: C, 15·3; H, 2·3; N, 3·5; P, 12·8%; total P/acid-labile P, 1·49/1. $(C_9H_{12}O_{15}N_2P_3)_2Ba_3,4H_2O$ requires C, 14·95; H, 2·2; N, 3·9; P, 12·85%; total P/acid-labile P, 1·5/1]. Phosphate analysis by Allen's method (*Biochem. J.*, 1940, **34**, 858) showed a negligible amount (0·25%) of orthophosphate to be present.

Identification of the Synthetic Uridine-5′ Triphosphate with a Specimen isolated from Yeast.—The synthetic sodium uridine-5′ triphosphate isolated by elution from a charcoal column (see above) and a sample isolated by anion-exchange from yeast extracts supplied by Dr. S. A. Morell were run side by side on Whatman No. 1 paper, using three solvent systems.

	Migration (cm.) (descending)		R_F (ascending)
	A	B	C
Synthetic uridine-5′ triphosphate	1·8	14·6	0·1
Natural uridine-5′ triphosphate	1·8	14·8	0·1
Synthetic barium uridine-5′ pyrophosphate	3·3	16·5	0·16
Barium uridine-5′ phosphate	8·0	20·0	0·20

Solvent systems: A, 95% ethanol (5)–M-ammonium acetate, pH 7 (2), run for 52 hr.; B, *iso*propanol (3)–1% ammonium sulphate solution (2), run for 20 hr. on paper previously soaked in 1% ammonium sulphate solution and dried; C, *n*-butanol (6)–acetic acid (2)–water (3).

Anion-exchange Chromatography of Uridine-5′ Triphosphate.—A solution of the lithium salt (1·5 g.) from preparation (*b*) above in water (200 c.c.) was brought on to a column (5 × 3 cm.) of Dowex-2 resin (chloride form). Uridine-5′ phosphate, uridine-5′ pyrophosphate and inorganic phosphate were eluted by a sodium chloride (0·1N)–hydrochloric acid (0·01N) solution (8 l.). Uridine-5′ triphosphate was then eluted by a sodium chloride (0·2N)–hydrochloric acid (0·01N) solution (7 l.), and finally with N-sodium chloride–0·02N-hydrochloric acid (1 l.). The combined eluates containing uridine-5′ triphosphate were adjusted to pH 7·5 with sodium hydroxide solution, diluted to 24 l., and passed through a column (0·5 × 6·2 cm.) of Dowex-2 resin (chloride form). The column was washed with 0·05N-lithium chloride (50 c.c.), and uridine-5′ triphosphate eluted with ice-cold N-hydrochloric acid (80 c.c.). The eluate was rapidly adjusted to pH 5 with N-lithium hydroxide and evaporated to 40 c.c. A saturated solution of barium acetate (0·4 g.) was added, followed by 0·18N-barium hydroxide solution to pH 6·0. The precipitate formed was centrifuged off, washed with water (2 × 20 c.c.), ethanol (2 × 30 c.c.), and ether (2 × 30 c.c.), and dried *in vacuo* over phosphoric oxide, yielding pure uridine-5′ barium triphosphate (498 mg.) as a stable white powder identical with the product obtained by charcoal chromatography. Addition of an equal volume of ethanol to the supernatant solution from the above precipitation gave a further precipitate which was similarly washed with water, ethanol, and ether and dried, giving a further quantity of the barium salt (148 mg.).

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