[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL]

Nucleoside Polyphosphates. II.[1] A Synthesis of Uridine-5'-di- and -Triphosphate[2a]

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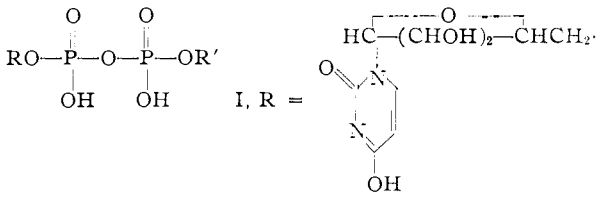
UDP[3] and UTP[3] have been synthesized by the treatment at room temperature of a mixture of UMP[3] and 85% orthophosphoric acid with excess of DCC[3] in aqueous pyridine. The procedures described make the biologically interesting uridine-5'-phosphates readily available in a pure state.

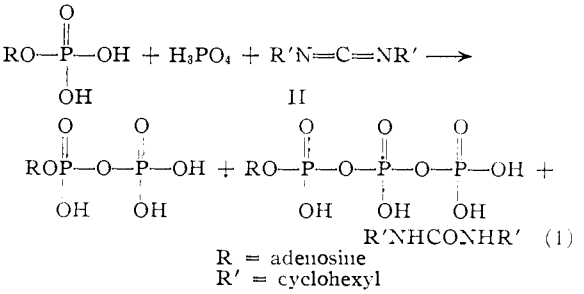
Whilst the chemistry and biochemistry of adenine nucleotides (AMP, ADP, ATP)[3] is of relatively long standing, interest, especially from the standpoint of their role in intermediary metabolism, in the analogous uridine compounds[3] is recent. The isolation of several UDP[3] derivatives by Park and Johnson,[4] and Park[4] was soon followed by the demonstration by Leloir's group of the coenzymic function of UDPG[3,5] in the enzymic interconversion[6] of galactose-1-phosphate and glucose-1-phosphate. More recent work reported from different laboratories has led to the recognition of the widespread occurrence of UDP-derived compounds[7] of the type I as well as UTP[8] and has



R', *e.g.*, a sugar residue.

also furnished extensive enzymic evidence for the biological importance of these substances.[9-11]

Recently a one-step synthesis (equation 1) of ADP[3] and ATP[3] by the treatment of a mixture of AMP[3] and 85% orthophosphoric acid in aqueous pyridine with excess of DCC[3] (II) was reported[12]



and there it was stated that the synthesis of other nucleoside di- and triphosphates by this new method was in progress. In this communication we wish to describe the preparation of UDP and UTP from UMP. The procedures detailed make these two substances readily available synthetically in a pure state for biochemical studies. Prior to the present work, chemical syntheses of the three uridine phosphates (UMP, UDP and UTP) have been achieved. UMP was first synthesized by Levene and Tipson,[13] in 1934, by Gulland and Hobday[14] in 1940 and by Michelson and Todd[15] in 1949. Two syntheses of UDP have been recorded by the Cambridge group,[16] who have also achieved a synthesis of UTP.[17]

In the previously described synthesis of ADP and ATP[12] and also in the study of the reaction of yeast ribonucleotides with DCC,[1,18] aqueous pyridine has proved to be a suitable solvent, a large excess of DCC being required. In the present work, the ready solubility of UMP in pyridine was advantageous; however, the incorporation of some water in the medium was necessary because of the relative insolubility of the pyridinium salt of orthophosphoric acid in pyridine alone.[19] Even so, in most

(1) This is paper VII in the series "Carbodiimides." Paper VI, Charles A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954).

(2a) Presented before the Division of Biological Chemistry at the 125th National Meeting of the American Chemical Society at Kansas City in March, 1954. (2b) Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

(3) The following abbreviations are used: UMP, uridine-5'-phosphate; UDP, uridine-5'-diphosphate; UTP, uridine-5'-triphosphate; AMP, adenosine-5'-phosphate (muscle adenylic acid); ADP, adenosine-5'-diphosphate; ATP, adenosine-5'-triphosphate; UDPG, uridine diphosphate glucose; DCC, dicyclohexylcarbodiimide.

(4) J. T. Park and M. J. Johnson, *J. Biol. Chem.*, **179**, 585 (1949); J. T. Park, *Federation Proc.*, **9**, 213 (1950); *J. Biol. Chem.*, **194**, 877, 885, 897 (1952).

(5) R. Caputto, L. F. Leloir, C. E. Cardini and A. C. Paladini, *ibid.*, **184**, 333 (1950).

(6) R. Caputto, L. F. Leloir and R. E. Trucco, *Enzymologia*, **12**, 350 (1948); J. F. Wilkinson, *Biochem. J.*, **44**, 460 (1949).

(7) A. C. Paladini and L. F. Leloir, *ibid.*, **51**, 426 (1952); E. Cabib, L. F. Leloir and C. E. Cardini, *J. Biol. Chem.*, **203**, 1055 (1953); J. G. Buchanan, V. H. Lynch, A. R. Benson, D. F. Bradley and M. Calvin, *ibid.*, 935 (1953); G. J. Dutton and I. D. E. Storey, *Proc. Biochem. Soc.*, **53**, XXXVII (1953); E. E. B. Smith and G. T. Mills, *Biochem. Biophys. Acta*, **13**, 386 (1954).

(8) (a) H. M. Kalckar, *ibid.*, **12**, 250 (1953); (b) A. Munch-Petersen, H. M. Kalckar, E. Cutolo and E. E. B. Smith, *Nature*, **172**, 1036 (1953); (c) H. M. Kalckar, B. Braganca and A. Munch-Petersen, *ibid.*, 1038; (d) R. Bergkvist and A. Deutsch, *Acta Chem. Scand.*, **7**, 1307 (1953); (e) H. Schmitz, V. R. Potter and R. B. Hulbert, *Proc. Am. Assoc. Cancer Research*, **1**, 47 (1953); (f) H. Schmitz, V. R. Potter, R. B. Hulbert and D. M. White, *Cancer Research*, **14**, 66 (1954); (g) S. H. Lipton, S. A. Morell, A. Frieden and R. M. Bock, THIS JOURNAL, **75**, 5449 (1953).

(9) A. Kornberg, "Phosphorus Metabolism," Vol. I, edited by W. D. McElroy and B. Glass, The John Hopkins Press, Baltimore, Md., 1951, p. 410; R. B. Hulbert *Federation, Proc.*, **12**, 222 (1953); H. Schmitz, R. B. Hulbert and V. R. Potter, *J. Biol. Chem.*, **209**, 41 (1954).

(10) L. F. Leloir and E. Cabib, THIS JOURNAL, **75**, 5445 (1953); L. F. Leloir and C. E. Cardini, *ibid.*, **75**, 6085 (1953).

(11) H. M. Kalckar [*Science*, **119**, 479 (1954)] has provided a useful summary of the main recent findings, large part of which has come from his group, concerning the enzymology of uridine phosphates.

(12) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954). This is paper 1 of the series "Nucleoside Polyphosphates."

(13) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **106**, 113 (1934).

(14) J. M. Gulland and G. I. Hobday, *J. Chem. Soc.*, 746 (1940).

(15) A. M. Michelson and A. R. Todd, *ibid.*, 2476 (1949).

(16) (a) N. Anand, V. M. Clark, R. H. Hall and A. R. Todd, *ibid.*, 3665 (1952); (b) G. W. Kenner, A. R. Todd and F. J. Weymouth, *ibid.*, 3675 (1952).

(17) Personal communication from Prof. A. R. Todd.

(18) Unpublished work of C. A. Dekker referred to in (1).

(19) In the few experiments carried out in methyl cyanide and dimethylformamide, in which solvents UMP is moderately and orthophosphoric acid freely soluble, to effect the condensation, unsatisfactory results were obtained.

experiments clear solution of all the reactants was not obtained for reasons already discussed.[20] The formation of UDP and higher uridine phosphates could be rapidly and conveniently followed by paper chromatography in the solvent system 1% ammonium sulfate–isopropyl alcohol (1:2, v./v.). Other solvent systems which are mentioned later (Table III) were used in conjunction to obtain further information on the nature of the reaction products. A more accurate method of routine analysis which was an adaptation[21,22] of Cohn and Carter's[23] ion-exchange procedure for the separation of adenosine polyphosphates was also used. An extensive study was made of the influence of the amount of phosphoric acid, the water content and the total volume of the solvent (pyridine) employed on the production of UDP, UTP and higher phosphates. Table I (experimental part) records the results obtained in some typical experiments. Results of further detailed study of the influence of time on the formation of uridine phosphates, under conditions which otherwise appeared to be most promising[24] (experiment 3 of Table I) are shown in Table II. The latter study showed clearly that the formation of UDP and UTP reached an optimum in relatively short time[25] and obviously longer reaction periods must result in larger proportion of higher phosphates and more complex "inorganic" phosphates.

The use of excess of phosphoric acid leads in the present method to the simultaneous formation of pyrophosphoric acid and probably one or more higher condensed "inorganic" phosphates. For the isolation of pure uridine phosphates three approaches have been tried. The adenine nucleotides[12] could be freed from the inorganic phosphates through two precipitations of their mercury salts at low *p*H. In the case of uridine phosphates, attempts along these lines, *e.g.*, selective precipitation at different *p*H's through the addition of mercuric, barium and magnesium ions, did not give satisfactory results. From the knowledge of the elution pattern of inorganic phosphates and uridine phosphates from Dowex 2 ion-exchange resin column it was clear that UTP could be eluted from such columns after UMP, UDP, orthophosphate and pyrophosphoric acid had been removed. Direct ion-exchange chromatography of the reaction mixture was therefore tried. However, the purity, as determined spectrophotometrically, of the samples of UTP isolated in this way did not exceed 50%. (In these experiments 6 molar excess of orthophosphoric acid had been used in the reaction with DCC). The third approach which was developed successfully and is described in detail in the experimental section consisted in the liberation of the free acids by passing the aqueous solution of pyridine salts of the reaction products through a Dowex-50 ion-exchange resin column and repeated precipitation of uridine phosphates with ether from ethanolic solution. The mixture of nucleotides was then separated on a Dowex-2 column, UDP and UTP being finally isolated as barium salts from the evaporated eluates. The above operations involved losses of the nucleotides; even so the final yield of UDP (20–25%) and UTP (*ca.* 20%) was fair, representing a 40–45% conversion of UMP to the desired phosphates.

The barium salt of uridine triphosphate as prepared in the present method has been assigned the formula $Ba_2(UTP)\cdot 4H_2O$ on the basis of two sets of elemental analyses. Spectrophotometric determination showed the sample to be 96% pure[26] with respect to this formula. The total phosphorus content and ratio of labile phosphorus (*i.e.*, phosphorus cleaved in 15 minutes by 1 *N* hydrochloric acid at 100°) to total phosphorus were determined by the method of Allen[27] and were in accord with the above formula. The synthetic sample was shown to be identical with uridine triphosphate, isolated from natural sources[28] (ref. 8g) from a comparison of their behavior on paper chromatograms developed in five different solvent systems (Table III). The synthetic sample of UDP was also shown to be homogeneous by extensive paper chromatography (Table III). The *mono* barium salt was prepared according to Kenner, *et al.*,[16b] and the molecular composition $[Ba(UDP)\cdot 3H_2O]$ already established for this salt was confirmed by elemental analysis and determination of the ratio of the total to labile phosphorus. Spectrophotometric estimation showed this sample to be 98% pure.

Experimental

Uridine-5'-phosphate.—Dibenzyl phosphite was prepared by the method of Atherton, Openshaw and Todd,[29] the product being subjected to short path distillation at 110–120° (0.1 mm.). The slightly modified method of Friedman, Klass and Seligman[30] has also been used successfully to give a highly pure crystalline product, distillation being unnecessary.

Dibenzyl phosphite (12 g., 0.0458 mole) was converted

(20) Footnote 26 in reference 12.

(21) Munch-Petersen, *et al.*, ref. 8b, have employed similar procedure for the separation of UTP from other UMP-derived compounds.

(22) We wish to express our thanks to Drs. V. R. Potter and R. B. Hulbert for communicating to us prior to publication the details of their refined method of ion exchange analysis of mixtures of uridine and adenosine polyphosphates.

(23) W. E. Cohn and C. E. Carter, THIS JOURNAL, **72**, 4273 (1950).

(24) An important consideration is the fact that the larger the proportion of phosphoric acid employed, the more complex is the nature of the "inorganic" phosphates concomitantly formed. On the other hand, as pointed out earlier,[12] an excess of phosphoric acid is desirable in order that the formation of the symmetrical diuridine-5'-pyrophosphate may be suppressed. Another consideration is the economy in the use of DCC; the conditions finally employed for the synthesis of uridine phosphates involved the use of much smaller quantities of DCC than those used in the ATP series.[12]

(25) Whilst the order of over-all concentration of reactants is very similar to that used previously[12] the reaction is much faster in the present series than in the ATP work.[12] The difference in speed is considered to be chiefly due to the solubility of UMP in pyridine and the higher pyridine–water ratio in the present experiments, the effective concentration of all the reactants in the reaction phase (pyridine) thus being much higher in the present experiments than in the parallel previous experiments.[12]

(26) The figure of 9900 for the molar extinction coefficient of UTP at *p*H 2 has been used (*cf.* ref. 8g.)

(27) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).

(28) We are grateful to Dr. R. M. Bock of the University of Wisconsin and Dr. S. A. Morell of Pabst Laboratories for supplying the sample of UTP isolated from yeast. Dr. Bock (private communication) stated and we confirmed through paper chromatography that the sample of natural origin was contaminated slightly by UDP and ATP.

(29) F. R. Atherton, H. T. Openshaw and A. R. Todd, *J. Chem. Soc.*, 382 (1945).

(30) O. M. Friedman, D. L. Klass and A. M. Seligman, THIS JOURNAL, **76**, 916 (1954).

to the corresponding phosphorochloridate[31] by the method of Kenner, Todd and Weymouth,[32] using 6.2 g. (0.0465 mole) of anhydrous N-chlorosuccinimide.[33] The clear benzene solution obtained after removal under suction of the insoluble succinimide on a dry sinter glass funnel was evaporated under reduced pressure (water pump) at *ca.* 10° using a receiver immersed in solid carbon dioxide–acetone-bath.

The preparation of UMP according to Michelson and Todd[15] has been modified, mainly with respect to the isolation procedure, to give a yield of over 80%. The concentrated solution of dibenzyl phosphorochloridate (*ca.* 20 cc.) as prepared above was added to a prechilled (in carbon dioxide–acetone-bath) solution of 4.5 g. (0.0158 mole) of dry 2′,3′-isopropylidene uridine[34] in 25 cc. of anhydrous pyridine, a further quantity of pyridine (10 cc.) being used to wash the residual phosphorylating agent into the reaction flask. The reaction flask was kept partly immersed in the cold bath, with exclusion of moisture, for three hours. (Pyridine hydrochloride may sometimes separate as a microcrystalline powder during this period.) The reaction mixture was then kept at room temperature for 16 hours when it colored to brown red. One cc. of water was then added and the solution was evaporated to a thick sirup which was maintained under suction at 50–60° for two hours. To the gum was added 150 cc. of 15% acetic acid solution and the mixture heated on a steam-bath for two hours with frequent agitation. The oil dissolved[35] to form a practically clear brown red solution. This was evaporated to a sirup which was dissolved in 25 cc. of water and the solution re-evaporated. The sirup was now dissolved in 200 cc. of 50% ethanol and after addition of 5 cc. of concentrated hydrochloric acid and 1.5 g. of 10% palladium–charcoal catalyst[37] was hydrogenated in a Parr hydrogenation apparatus at *ca.* 12 p.s.i. Hydrogenation was complete in 24–36 hours[38] and after removal of the catalyst, the filtrate was evaporated to a gum which was freed from hydrochloric acid by two evaporations of its aqueous solution. The gum was dissolved in water (*ca.* 20 cc.) and passed through two Dowex-50 ion-exchange resin (200–325[39] mesh, hydrogen form) columns (8 cm. long × 3 cm. diameter) and the columns washed with water until the *p*H of the effluent rose to neutrality. The combined eluate which contained mixture of free UMP and orthophosphoric acid was evaporated at 25° under reduced pressure to a sirup which was kept under suction in a high vacuum for an hour. It was then taken up in 10 cc. of acetone and UMP precipitated as an oil by the addition of 150 cc. of anhydrous ether. The ethereal layer was sucked off and the precipitation process repeated four times by dissolving the oil obtained every time first in 5–8 cc. of ethanol and then adding ether under agitation (the ethereal layer was clarified by centrifugation). UMP was finally obtained as a hard gum and was practically free from orthophosphoric acid.[40] It was dissolved in 15 cc. of water, the solution neutralized with 2 *N* lithium hydroxide and the barium salt precipitated by the addition of 12 cc. of 2 *M* barium acetate solution and 60 cc. of ethanol. It was collected by centrifugation, washed thoroughly twice with 100-cc. portions of 50% ethanol, then ethanol and ether; wt. of air dried material 7.05 g. Spectrophotometric determination, assuming the figure of 9900 for the molar extinction coefficient[26] of UMP at 260 mμ, showed that this hydrated salt contained a total of 4.15 g. of UMP (yield 81%).

The Reaction of a Mixture of UMP and Phosphoric Acid with DCC. General Method.—DCC[42] was added to a solution of UMP and 85% phosphoric acid in aqueous pyridine and the non-homogeneous mixture was agitated mechanically. Crystals of dicyclohexylurea began to separate in 10–20 minutes. After being agitated for varying lengths of time, the reaction mixture was diluted with water, under cooling, and filtered from the urea which was washed thrice with small amounts of water. The combined filtrate and washings were extracted repeatedly with ether to remove excess of pyridine, the residual aqueous solution being freed from dissolved ether through suction under agitation. An aliquot of the solution containing an equivalent of approximately 2 mg. of UMP originally employed was adjusted to *p*H 7 with ammonium hydroxide and applied on a Dowex-2 ion-exchange resin (200–325 mesh,[39] chloride form) bed (1.5 cm. long × 1 cm. diameter). After washing the column with approximately 20 cc. of water, which removed pyridine, uridine phosphates were eluted as below, the eluant being changed when optical density of the effluent at 260 mμ fell below 0.050. Average flow rate of liquid was 1 cc. per minute: UMP with 0.01 *N* HCl + 0.015 *M* NaCl (40–50 cc.); UDP, 0.01 *N* HCl + 0.1 *M* NaCl (70–80 cc.); UTP, 0.01 *N* HCl + 0.2 *N* NaCl (80–90 cc.); higher phosphates, 1 *N* HCl (*ca.* 40 cc.). Table I records the results of ion-exchange analysis[43] (followed by determination of the total optical density of the respective eluates) of reaction products obtained in some typical experiments. Table II

TABLE I

REACTION OF A MIXTURE OF UMP AND PHOSPHORIC ACID WITH DCC[a,b]

Phosphoric[c] acid	DCC[c]	Pyridine–water ratio	Solvent, cc.	Yield, % UMP	UDP	UTP	Higher[d] phosphates
10	40	5	12	41	19	40	..
6	45	15	11	15	16	55	14
4	20	15	8.2	17	22	40	21
4	30	15	6.1	11	23	36	29
2	20	15	6.2	19	32[e]	31	18

[a] Approximately 100 mg. of UMP was used in every experiment. [b] The time of reaction was 6 hours in every experiment. In view of later work, it is likely that the formation of UDP and UTP reached an optimum in shorter time in some experiments. [c] The figures under these columns represent the number of molecular proportions (with respect to UMP used) of these reactants used in individual experiments. [d] These are presumably the linear phosphates (*e.g.*, uridine-5′-tetraphosphate) which are eluted with 1 *N* hydrochloric acid. [e] This figure includes perhaps some of the symmetrical P¹:P² di-uridine-5′-pyrophosphate.

(31) New nomenclature of phosphorus compounds adopted by the International Union of Pure and Applied Chemistry; see *e.g.*, *J. Chem. Soc.*, 5122 (1952).

(32) G. W. Kenner, A. R. Todd and F. J. Weymouth, *ibid.*, 3675 (1952).

(33) Eastman Kodak Technical Product was extracted for 18 hours in a Soxhlet apparatus with dry carbon tetrachloride. The material remaining in the extraction thimble (*ca.* 60–65%) was pure (m.p. 150–152°) and was directly used.

(34) This was prepared from a commercially available sample of uridine by the method of Levene and Tipson.[13] After removal of acetone, the product was crystallized from methanol containing some petroleum ether (b.p. 30–60°). Before use, it was dried at 110° for 18 hours in a vacuum.

(35) This step removes the isopropylidene group and one benzyl group to form uridine-5′-monobenzyl phosphate (*cf.* the preparation of adenosine-5′-benzyl phosphate from 2′,3′-isopropylidene adenosine-5′-dibenzyl phosphate[36]).

(36) D. M. Brown, L. J. Haynes and A. R. Todd, *J. Chem. Soc.*, 3299 (1950).

(37) R. Mozingo, *Org. Syntheses*, **26**, 78 (1946).

(38) The progress of hydrogenation can be followed by direct examination of the aqueous-alcoholic solution on paper chromatograms developed in 1% ammonium sulfate:isopropyl alcohol (1:2, v./v.); UMP travels much slower than uridine-5′-monobenzyl phosphate.

(39) Prepared from the commercially available 200–400 mesh sample by sieving through 200- and 325-mesh sieves.

(40) The complete removal of orthophosphoric acid may be tested by paper chromatography in solvent system 2 (Table III) and subsequently treating the paper chromatograms according to Hanes and Isherwood[41]; orthophosphate spot becomes visible immediately after spraying with the molybdate reagent as yellow, turning blue after heating and reduction in hydrogen sulfide atmosphere.

(41) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(42) DCC was prepared as described previously.[12] The use of hypochlorite in the oxidation of *s*-dicyclohexylthiourea (E. Schmidt, M. Seefelder, R. G. Jennen, W. Strewsky and H. Von Marius, *Ann.*, **571**, 83 (1951), has been re-examined. The product obtained by this method is contaminated with some colored impurity but is adequate for use in the synthetic work. This method is preferred for large scale (around 100 g.) preparation of DCC.

(43) It is known (see *e.g.*, ref. 44 and 23) that orthophosphoric acid is eluted along with UMP and it was separately established that pyrophosphoric acid can be eluted with 0.01 *N* HCl + 0.1 *N* NaCl. Higher condensed inorganic phosphates are eluted along with UTP and later. It is considered that the presence of the "inorganic" phosphates did not interfere with the analysis of uridine phosphates.

(44) W. E. Cohn and E. Volkin, *J. Biol. Chem.*, **203**, 319 (1953).

shows the results of the study of the influence of time, under the conditions of experiment 3 (Table I), on the relative proportions of uridine phosphates.

TABLE II

Time,[a] hr.	Yield, % UMP	UDP	UTP	Higher phosphates
1	14.8	30.1	44.0	11.1
2	14.8	25.6	44.2	15.4
3	14.3	24.0	45.1	16.6
4	15.4	24.7	41	18.9
7	14.1	21.8	40.1	24.0

[a] The presence of an excess of DCC during the reaction periods is assumed. Some unreacted DCC was found to be present during working up in the last experiment.

Isolation of UDP and UTP.—Five hundred mg. of UMP was liberated from an appropriate amount of the barium salt by passing a solution of the latter in dilute acetic acid through a Dowex-50 ion-exchange resin column (5 cm. long × 2 cm. diameter) and complete removal under suction of water and acetic acid; the removal of the latter is aided by repeated evaporation after addition of water to the residue. To the solution of UMP and 85% orthophosphoric acid (720 mg.) in a mixture of water (2.45 cc.) and pyridine (36.5 cc.) was added 6 g. of DCC and the mixture was vigorously shaken mechanically for two hours. It was then diluted with 25 cc. of water under cooling and filtered under suction from dicyclohexylurea which was washed with three portions of water (total volume, 20 cc.). The combined filtrate and washings were extracted six times with 100-cc. portions of ether, the aqueous solution then freed from dissolved ether under agitation and concentrated to *ca.* 15 cc. at 10–15° in a vacuum. The concentrate was passed in two equal parts through two Dowex-50 ion-exchange resin (200–325 mesh, hydrogen form) columns (6 cm. long × 2 cm. diameter), the columns being washed with water until the *p*H of the effluents rose to neutrality. The combined eluate which was collected in flasks chilled in an ice–salt-bath, was freeze-dried, the flask containing the frozen solution being kept immersed in ice-water throughout. The residual gum was dissolved at below 0° in 10 cc. of ethanol and transferred to a 200-cc. centrifuge tube. The gum and the cloudy ethereal solution resulting on the addition of 100 cc. of cold anhydrous ether were centrifuged at 0° and the clear ethereal solution poured off. The process of precipitation from ethanol–ether mixture was repeated three times, using first 10 cc. of ethanol and 100 cc. of ether, then 15 cc. of ethanol and 100 cc. of ether in the next two precipitations. All the operations were performed as far as possible at or below 0°. The partly solid gum finally obtained was dissolved in 5 cc. of cold water, the solution rapidly neutralized with *N* sodium hydroxide solution and stored at −20° until required. Paper chromatography in the solvent systems 1 and 2 (Table III) showed this solution to be free from orthophosphoric, pyrophosphoric and metaphosphoric acids (the chromatograms being treated according to Hanes and Isherwood[41,45] for the location of various phosphates). The solution of the sodium salts of uridine phosphates was absorbed on the top of a Dowex-2 ion-exchange resin (200–325 mesh, chloride form) column (7 cm. long × 2 cm. diameter). After a water (100 cc.) wash, which did not remove any appreciable amount of ultraviolet absorbing material, UMP was removed with 0.01 *N* HCl + 0.015 *M* NaCl solution (total volume 490 cc., optical density at 260 mμ 1.7). UDP was then eluted with 0.01 *N* HCl + 0.1 *M* NaCl, a flow rate of approximately 4 cc. per minute being maintained. Six hundred and sixty cc. of eluate with optical density of 5.5 at 260 mμ was collected before optical density fell below 0.5, the next 300 cc. of the effluent being discarded. UTP was eluted next with 0.01 *N* HCl + 0.25 *M* NaCl solution, 520 cc. of eluate with optical density 6.2 being collected.

The neutralized eluate containing UTP was evaporated to complete dryness at 10–15° *in vacuo* (8–10 mm. pressure), the last 20 cc. of water being removed at 0° in a high vacuum. The dry residue was transferred to a fritted glass funnel and extracted with three 2-cc. portions of water, the extracts being collected through filtration under suction. (The residual sodium chloride cake did not contain any appreciable amount of ultraviolet absorbing material). To the combined extracts was added 2 cc. of 1 *M* barium acetate solution and the precipitated barium salt centrifuged off after being kept at 0° for 6 hours. It was washed once with one-half cc. of water (the washing being added to the mother liquor), twice with 5 cc. of 50% ethanol, then ethanol and ether, wt. 160 mg. The analyses reported below were carried out on this sample. A further amount (60–80 mg.) of practically pure barium salt of UTP was obtained on addition of 4 cc. of ethanol to the above mother liquor, the barium salt being washed thoroughly with 50% ethanol to redissolve some sodium chloride which also was precipitated on the addition of ethanol. After being kept in a vacuum over phosphorus pentoxide at room temperature the main sample was submitted for analysis.

TABLE III

R_F VALUES OF URIDINE PHOSPHATES AND INORGANIC PHOSPHATES

Solvent system	R_f values UMP	UDP	UTP	Higher P	Ortho-P	Pyro-P	Meta-P
(1) 1% ammonium sulfate–isopropyl alcohol, 1:2 (v./v.)[16,48]	0.51	0.34	0.23	0.17	0.55	0.28	0.16
(2) Isopropyl alcohol (75 cc.)–water (25 cc.)–trichloroacetic acid (5 g.)–ammonia 0.25 cc. (sp. gr., 0.9)[49]	.36	.14	.04	..	.67	.30	.13
(3) Isobutyric acid (100 cc.)–*N* ammonium hydroxide (60 cc.)–0.1 *M* ethylenediaminetetraacetic acid (1.6 cc.)[50,51] disodium salt	.34	.25	.19	.14	.42	.30	.20
(4) Ethanol (75 cc.)–1 *M* ammonium acetate (30 cc.)[8g]	.11	.053	.035	0	.11	0	0
(5) 5% aqueous disodium hydrogen phosphate–*n* hexyl alcohol[8g]	.85	.91	.92	.92	..	..	..

Anal. Calcd. for $C_9H_{11}N_2O_{15}P_3Ba_2 \cdot 4H_2O$: C, 13.05; H, 2.31; N, 3.36. Calcd. for $C_9H_{11}N_2O_{15}P_3Ba_2 \cdot 3H_2O$: C, 13.33; H, 2.1; N, 3.45; P, 11.2. Found[46,47]: C, 13.22, 13.0; H, 2.76, 2.3; N, 3.18, 3.3; P, 11.7. Ratio of easily hydrolyzable phosphorus (in 15 minutes at 100° with 1 *N* hydrochloric acid) to total phosphorus,[23] 2:3. 19.8 mg. of the barium salt was dissolved in 25 cc. of 0.01 *N* HCl

(45) This method has been found to be completely satisfactory for the location on paper chromatograms of all inorganic and organic polyphosphates which are capable of readily releasing orthophosphate.

(46) One set of analyses was performed by Mr. W. Manser of Zürich, Switzerland, and the second set by Mr. V. Tashinian, University of California, Berkeley.

(47) An excess of sulfate ions present in samples prepared for chromatography will give a blue spot (Hanes and Isherwood) which has an R_f value identical with pyrophosphoric acid in solvent system II.

(48) The chromatographic papers (Whatman No. 1) were previously soaked in 1% aqueous ammonium sulfate solution and dried.

(49) J. P. Ebel, *Bull. soc. chim. (France)*, 991 (1953).

(50) H. A. Krebs and R. Hems, *Biochem. Biophys. Acta*, **12**, 172 (1953). *Cf.*, R. Zetterström and M. Ljunggren, *Acta Chem. Scand.*, **5**, 291 (1951).

(51) The chromatographic papers were previously washed as described by L. V. Eggleston and R. Hems, *Biochem. J.*, **52**, 156 (1952).

and a 2-cc. aliquot diluted further to 25 cc. Optical density at 260 mμ using a Beckman spectrophotometer (model DU) was found to be 0.720; from this, 18.9 mg. of $Ba_2UTP \cdot 4H_2O$ was calculated[26] to be present in the original 25 cc. of solution, the synthetic sample being thus 95.5% pure. Ion exchange analysis showed only a trace (<2%) of UDP to be present. The synthetic sample was free from "inorganic" phosphates[47] and on paper chromatograms developed in five different solvent systems (Table III) migrated as a single spot.

The procedure for the isolation of UDP was identical with that described above for UTP, except for addition of 6 cc. of ethanol for the precipitation of the barium salt. The concomitantly precipitated sodium chloride was removed during washings with 50% ethanol; wt. of the barium salt, 195 mg. 50 mg. of this sample was dissolved at 0° in ice-cold 0.05 *N* hydrochloric acid and the *mono* barium salt ($C_9H_{12}O_{12}N_2PBa \cdot 3H_2O$)[16] precipitated with equal volume of cold ethanol, collected by centrifugation and washed with ethanol and ether; yield 36 mg. After being exposed to the air for 24 hours, the sample was submitted for analysis.[48] *Anal.* Calcd. for $C_9H_{12}O_{12}N_2Ba \cdot 3H_2O$: C, 18.2; H, 3.0; N, 4.7; P, 10.4. Found: C, 18.7; H, 3.3; N, 4.3; P, 10.8. Ratio of labile P to total P, 1:2. The synthetic sample was found to be homogeneous on paper chromatography in five different solvent systems (refs. in Table III) and was free from "inorganic" phosphates.[47] Spectrophotometric estimation carried out as described for the barium salt of UTP showed this sample to be 98% pure with respect to the above formula.

(48) Analysis by Mr. V. Tashinian, University of California, Berkeley.

Paper Chromatography of Uridine Phosphates and "Inorganic Phosphates."—Paper chromatography was used throughout the present work; it was especially useful in following the removal of "inorganic phosphates" from the nucleotides. The solvent systems found most useful were 1 and 2 (Table III), descending technique being used. The solvent systems 3–5 of Table III (ascending technique) were used to confirm the purity and identity of the synthetic nucleotides. Standards were always run side by side since the R_f values varied somewhat with time.

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[CONTRIBUTION FROM THE DEPARTMENTS OF NEUROLOGY AND MICROBIOLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL]

Immunochemical Studies on Blood Groups. XV. The Effect of Mild Acid Hydrolysis on the Glucosamine and Galactosamine in Blood Group Substances[1]

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The glucosamine and galactosamine contents of the dialyzable and non-dialyzable portions of blood group substances which had been subjected to mild acid hydrolysis have been studied. With all of these substances the non-dialyzable fractions showed lower glucosamine–galactosamine ratios than did the original blood group substances. The hexosamines of the dialyzable fractions were strikingly related to blood group activity for the hog and human substances, showing glucosamine–galactosamine ratios of 2 to 4 for the A substances, 13–14 for the O (H) substances; only glucosamine was split off from human B substances. Horse and bovine substances appeared to exhibit species rather than blood group specificity with respect to the splitting off of these hexosamines.

Introduction

It has been known for some time[2] that treatment of blood group substances for two hours at 100° in dilute hydrochloric acid at a *p*H of 1.5–2.0 resulted in almost complete destruction of blood group activity as measured by hemagglutination inhibition tests. At the same time a striking increase in reactivity with type XIV anti-pneumococcal horse serum developed. These changes have been shown to occur in hog,[2] human,[2] horse[3] and cattle[4] substances with A, B or O (H) activity as well as with inactive substances of similar chemical composition. Study of the chemical changes in hog A and O (H) preparations associated with this procedure showed that 60–80% of the fucose together with small amounts of hexosamine, galactose, amino acid nitrogen and oligosaccharide had become dialyzable. The non-dialyzable material, when reisolated by alcohol precipitation, was found to have a substantially lower fucose content but otherwise was very similar to the original material. Mild acid hydrolysis with 1 *N* acetic acid was also shown by Aminoff, Morgan and Watkins[5] to produce similar changes and to result in increased Forssman activity of the A substance. By means of the recently described method[6] for the separation and determination of glucosamine and galactosamine, a re-examination of this reaction was undertaken to see whether more information could be obtained on the chemical changes produced by mild acid hydrolysis and to relate them to immunological specificity.

Method.—Measured amounts (5–20 mg.) of the materials to be studied were dissolved in a few ml. of water and adjusted to *p*H 1.6 with hydrochloric acid in test-tubes which were then sealed with rubber caps. After immersion in a boiling water-bath for various times (care being taken to release the pressure by puncturing the caps with hypodermic needles), the contents were quantitatively transferred to well washed sausage casings and dialyzed against at least four 25-fold portions of distilled water changed at daily intervals. The dialyzates (dial.) were combined, evaporated down under reduced pressure (water-bath *ca.* 50°), taken up in 2 *N* hydrochloric acid and hydrolyzed at 100° for two hours. The non-dialyzable material (P-1) was transferred from the casing, made up to 2 *N* with concentrated hydrochloric acid and similarly hydrolyzed at 100° for two hours. Both the dialyzable and non-dialyzable fractions were then analyzed for glucosamine and galactosa-

(1) This investigation was carried out under grants from the National Institutes of Health, Public Health Service (RG-34) and the William J. Matheson Commission.

(2) E. A. Kabat, H. Baer, A. E. Bezer and V. Knaub, *J. Exp. Med.*, **88**, 43 (1948).

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(4) S. M. Beiser and E. A. Kabat, *J. Immunol.*, **68**, 19 (1952).

(5) D. Aminoff, W. T. J. Morgan and W. M. Watkins, *Biochem. J.*, **43**, xxxvi (1948); **46**, 426 (1950).

(6) S. Leskowitz and E. A. Kabat, THIS JOURNAL, **76**, 4878 (1954)