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The Isolation of Uridine-5′-Triphosphate from Muscle

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The isolation of uridine-5′-triphosphate from rabbit skeletal muscle is described. The identification is based on the results of paper chromatography and ultraviolet absorption measurements on the products of hydrolysis, together with enzymic evidence and chemical analysis, electrometric titration and ultraviolet absorption spectra at different pH values, of the compound before hydrolysis.

The occurrence of ribonucleosides in the form of polyphosphate derivatives — as distinct from that in nucleic acids — was first established for adenosine, by the isolation of adenosine triphosphate (ATP) from muscle[1, 2]. Similar derivatives of the other ribonucleosides, present in nucleic acids, were not known until the isolation of uridine-diphosphate-glucose (UDPG) by Caputto, Leloir, Cardini and Paladini[3], followed by the demonstration of other derivatives[4, 5, 6] of uridine diphosphate (UDP) in biological material. The natural occurrence of uridine triphosphate (UTP), previously obtained from UDP[7] and UDPG[8] in enzymic reactions, has been established by its isolation from muscle[9]; moreover, UTP has been isolated from yeast[10] and demonstrated in various tissues[11].

UTP from muscle was first obtained by ion-exchange chromatography of ATP preparations originating from rabbit skeletal muscle. Ion-exchange chromatography of ATP on Dowex-1 or Dowex-2 chloride columns, using the chloride elution system according to Cohn and Carter[12], gave regularly a contaminating phosphate fraction amounting to 2—4 % of the ATP and appearing in the effluent by elution with *N* hydrochloric acid, after the ATP had been removed from the column by the eluting solvent of next lower anion concentration. Phosphorus analysis showed the presence of acid-labile phosphate amounting to two-thirds of the total phosphorus indicating a triphosphate of ATP type. Ultraviolet absorption measurements showing a maximum around 260 mμ, suggested the presence of a nucleoside derivative.

By application of the ion-exchange procedure directly to trichloroacetic acid extracts of muscle, similar amounts of the unknown triphosphate fraction were obtained.

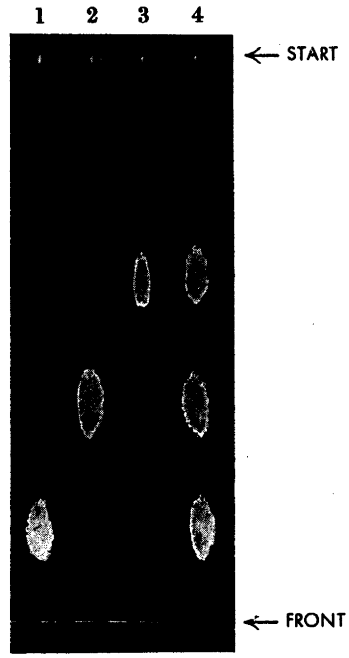

Fig. 1. Separation of ATP, GTP and UTP by paper chromatography in saturated ammonium sulphate-water-isopropanol (79:19:2), 10 hours. (1) UTP; (2) GTP; (3) ATP; (4) mixture of ATP, GTP, and UTP; approx. 50 μg of each compound.

Paper chromatography in the saturated ammonium sulphate solution-water-*iso*-propanol (79 : 19 : 2) solvent system resolves the unknown triphosphate material into two components, both triphosphates, according to phosphorus analysis, and later identified as UTP and guanosine triphosphate (GTP). The same solvent system is equally suitable for the separation of mixtures containing ATP besides GTP and UTP (Fig. 1). A separation of GTP and UTP can also be achieved by fractional reprecipitations of the mixture of barium salts, as obtained from pooled fractions of the effluent from ion-exchange columns. Ion-exchange chromatography on Dowex-1 formate in the formate system[13], however, is the method which has been used by preference for the preparation of the pure triphosphates in quantity.

The first indication as to the nature of the triphosphate ultimately identified as UTP, was obtained by measurement of the ultraviolet absorption spectra in acid and in alkali, resulting in absorption curves typical for uridine derivatives. Subsequent hydrolysis in 70 % perchloric acid for 1 hour at 100° C, followed by paper chromatography, gave one ultraviolet absorbing spot with the same R_F value as uracil, chromatographed side by side with the experimental solution in four different solvent systems (Table 1). The ultraviolet absorption curves in acid and in alkali were likewise identical with those of uracil (Fig. 2).

Hydrolysis of UTP in *N* hydrochloric acid for 1 hour at 100° C resulted in the splitting off of two-thirds of the bound phosphorus in the form of ortho-phosphate under formation of a nucleoside monophosphate, in agreement with the expected behaviour of a pyrimidine nucleoside-5′-polyphosphate. Paper

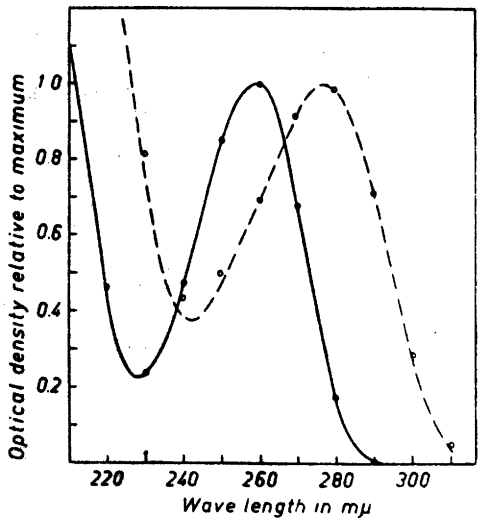

Fig. 2. Ultraviolet absorption curves for uracil obtained from UTP by hydrolysis in 70 % perchloric acid (1 hour at 100 °C) in comparison with authentic uracil in 0.1 N acid and 0.1 N alkali. ——— *uracil in acid and* — — — *in alkali.* ● *uracil from UTP in acid and* ○ *in alkali.*

chromatography of the hydrolysis products gave an ultraviolet absorbing spot with the R_F value of 5′-uridylic acid (UMP) in four different solvent systems (Table 1). Identity with UMP was likewise demonstrated by comparison of the ultraviolet absorption curves in acid, in alkali and after treatment with bromine,

Table 1. R_F values of the ultraviolet-absorbing base obtained from UTP by hydrolysis in 70 % perchloric acid (1 hour at 100 °C) in comparison with uracil, and of the nucleotide obtained by hydrolysis in N hydrochloric acid (1 hour at 100 °C) in comparison with 5′-UMP on paper chromatography. Solvent systems: (A) hydrochloric acid (sp.gr. 1.180)-isopropanol-water (17:65:18); (B) n-butanol-formic acid (sp.gr. 1.220)-water (77:10:13); (C) n-butanol-ammonia (sp. gr. 0.880)-water (84:5:14); (D) 5 % potassium dihydrogen phosphate in isoamyl alcohol. Descending chromatography was used in systems A, B and C, and ascending chromatography in D.

	R_F			
	A	B	C	D
Ultraviolet absorbing spot from UTP	0.68	0.40	0.19	0.72
Uracil	0.68	0.39	0.19	0.72
Nucleotide from UTP	0.79	0.03	0	0.84
5′-UMP	0.79	0.03	0	0.84

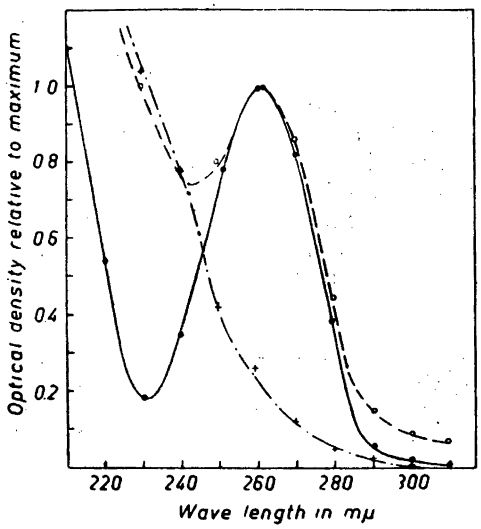

Fig. 3. Ultraviolet absorption curves for UMP obtained from UTP by hydrolysis in N hydrochloric acid (1 hour at 100 °C) compared with those for 5'-UMP in 0.1 N acid, in 0.1 N alkali and after treatment with bromine. ———— *5'-UMP in acid,* — — — *in alkali and* —·—·— *after treatment with bromine.* ● *UMP from UTP in acid,* ○ *in alkali and* + *after treatment with bromine.*

followed by aeration (Fig. 3). Location of the phosphate group in the 5'-position of UMP derived from muscle UTP was confirmed by experiments with 5'-nucleotidase[14] from snake venom (Table 2).

Pentose estimations with the orcinol reagent[15] on UTP before and after treatment with bromine[16] show colour development only in the latter case, suggesting the presence of pyrimidine-bound pentose. Hydrolysis of UTP for 2 hours in 2 *N* hydrochloric acid after treatment with bromine and paper

Table 2. Action of 5'-nucleotidase[14,6] on UMP obtained from UTP. 1 μmole nucleotide in 0.1 ml M *glycine buffer (pH 8.5) + 0.1 ml 0.1* M *magnesium chloride + 0.1 ml of a solution of Crotalus adamanteus venom (2 mg/ml) in a total volume of 1 ml were incubated for 45 minutes at 37 °C. Phosphorus was estimated after addition of 1 ml ethanol and centrifugation.*

Compound (1 μmole)	Orthophosphate in μmoles		μmoles orthophosphate formed
	time of incubation		
	0 min.	45 min.	
Adenosine-5'-phosphate	0.01	0.92	0.91
Adenosine-3'-phosphate	0.005	0.005	0
Uridine-3'-phosphate	0.007	0.013	0.006
Uridine phosphate from UTP	0	0.98	0.98

chromatography of the resulting solution in the *n*-butanol-acetic acid-water (4 : 1 : 5) system, gave a spot, indistinguishable from that obtained from D-ribose ($R_F = 0.32$) on development with aniline hydrogen phthalate.

The identification of uracil, D-ribose and the 5′-UMP moiety establishes the UTP isolated from muscle as a derivative of 5′-UMP. Chemical analysis (base : pentose : total phosphorus : acid labile phosphorus) shows it to be a triphosphate, containing two acid-labile phosphate groups attached to 5′-UMP.

Experiments on copper complex formation show that UTP forms a copper complex in analogy to other 5′-substituted nucleoside compounds[17] without substituents in the 2′- and 3′-positions of the molecule (Table 3).

Table 3. Copper complex formation[17,3]. *To 1 μmole of the sample, about 3 mg of copper phosphate and 0.5 ml of 20 % trisodium phosphate were added. The suspension was centrifuged after 90 minutes and the supernatant mixed with 1 ml of 0.5 % sodium diethyl dithiocarbamate. The copper complex was extracted with 6 ml amyl alcohol and measured in a Klett-Summerson colorimeter with filter No. 42.*

Compound (1 μmole)	Colorimeter reading
Adenosine-3′-phosphate	23
Adenosine-5′-phosphate	330
Adenosine-5′-triphosphate	320
Uridine-3′-phosphate	26
Uridine	333
Uridine triphosphate	360

Similar results were obtained in experiments on the oxidation of UTP with periodate, giving evidence of unsubstituted 2′- and 3′-hydroxyl groups in the ribose moiety of the molecule, under conditions where no splitting off of the labile phosphate groups takes place (Table 4). The results of periodate oxidation provide at the same time a quantitative measure of the pentose content of UTP.

The results of copper complex formation and periodate oxidation show that the two labile phosphate groups present in UTP cannot be attached to 5′-UMP in the 2′- and 3′-positions. Although location of the labile phosphate groups in the pyrimidine part of the molecule is not excluded by these experiments, the rates of acid hydrolysis (Fig. 4) as compared with ATP, make the formulation of UTP as a 5′-triphosphate most likely. Furthermore, electrometric titration of UTP shows the presence of three primary and one secondary phosphate groups, in harmony with the expectations for a 5′-triphosphate (Fig. 5).

Finally, comparison of the reaction[19] with actomyosin gel of UTP prepared from muscle with that of synthetic[20] 5′-UTP shows complete identity of the two compounds both in regard to dephosphorylation of UTP to the diphosphate and volume constriction of the actomyosin gel.

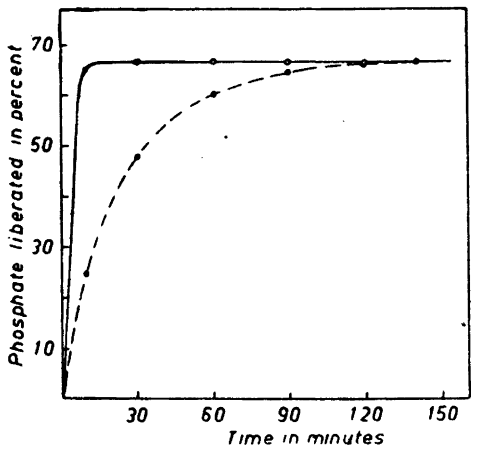

Fig. 4. Acid hydrolysis of UTP at 100 °C. ———— in N hydrochloric acid and — — — in 0.1 N hydrochloric acid.

Table 4. Periodate oxidation.[18] 0.50 μmole potassium metaperiodate was added to approx. 0.3 μmole of the sodium salt of the nucleotide in a total volume of 3 ml. The amount of periodate consumed was measured at 227 mμ against a blank containing periodate of the same concentration.

Compound	μmoles periodate		μmoles periodate consumed per μmole nucleotide
	added	consumed	
Adenosine-3′-phosphate, 0.31 μmole	0.50	0.04	0.13
Adenosine-5′-phosphate, 0.27 μmole	0.50	0.26	0.96
Adenosine-5′-triphosphate, 0.30 μmole	0.50	0.29	0.97
Uridine-3′-phosphate, 0.33 μmole	0.50	0.03	0.09
Uridine triphosphate, 0.36 μmole	0.50	0.36	1.00

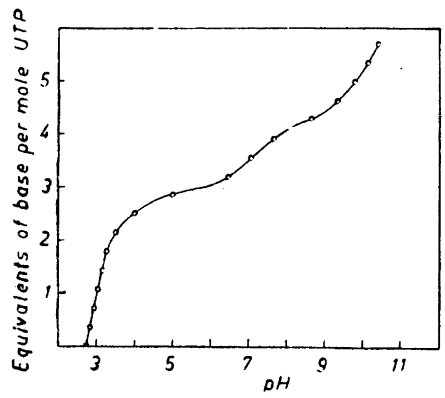

Fig. 5. Electrometric titration of UTP. 5 μmoles UTP titrated with 0.10 N sodium hydroxide.

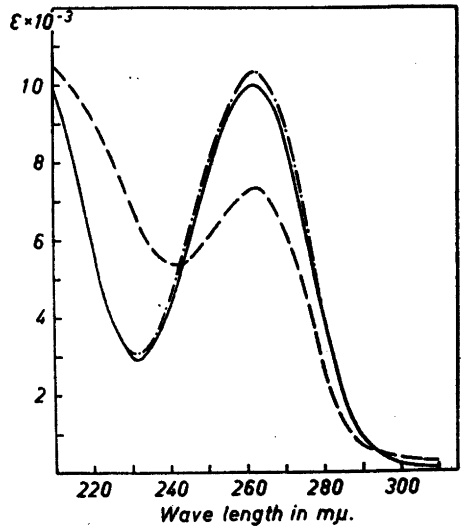

Fig. 6. Ultraviolet absorption curves for UTP from muscle. ——— *at pH 1.0,* — — — *at pH 6.5 and* —·—·— *at pH 11.0.*

On the basis of the evidence obtained, we feel justified in formulating UTP isolated from muscle as uridine-5′-triphosphate:

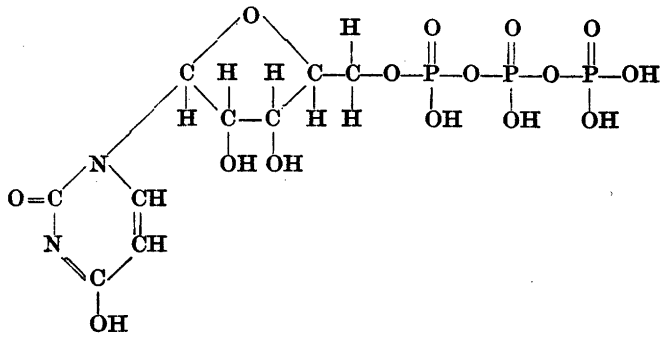

Fig. 6 gives the ultraviolet absorption curves for 5′-UTP obtained from muscle and purified by ion-exchange chromatography. Molar extinction coefficients of 10 050 at pH 1.0, 7 330 at pH 6.5 and 10 350 at pH 11.0 were obtained at 262 mμ (± 2 %).

EXPERIMENTAL

Isolation of UTP. ATP, used as the starting material for the preparation of UTP, was obtained as the barium salt from the muscles of the hind limbs and the back of rabbits by the method described by D. M. Needham[21]. In several experiments, the crude barium salt obtained from the neutralized trichloroacetic acid extract of muscle in the first step of the preparation, was used directly.

For ion-exchange chromatography, the barium salt was converted to the free acid with Dowex-50 resin in the hydrogen form[22] and absorbed from dilute ammoniacal solution at about pH 11 on columns of the anion-exchange resins Dowex-1 or Dowex-2 (250—500 mesh) in the chloride form. Columns of bed size 8 cm^2 × 30 cm were used for amounts of approx. 500 mg of total phosphorus, with a flow rate of 4 ml/min. The eluting solvents of Cohn and Carter[12] were employed, 1) 0.003 *M* hydrochloric acid, 2) 0.02 *M* sodium chloride in 0.01 *M* hydrochloric acid, 3) 0.2 *M* sodium chloride in 0.01 *M* hydrochloric acid and 4) *M* hydrochloric acid. Fractions of 100 ml were taken with an automatic fraction collector adjustable to different time intervals and the fractions were analysed for total phosphorus or/and by optical density measurements. For the isolation of the nucleoside triphosphate fraction appearing in the *M* hydrochloric acid effluent, appropriate fractions of this effluent were pooled and the barium salt precipitated by addition of a small excess of 2 *N* barium acetate solution to the neutralized pooled fractions at 0 °C, followed by 1—2 volumes of ethanol in some experiments, when rapid precipitation of the barium salt was desired.

2—8 (mostly 4—8) mg (calc. as the free acid) of nucleoside triphosphate beside 100—200 (mostly 150—200) mg ATP were obtained from 100 g muscle in different experiments.

For the preparation of the nucleoside triphosphate fraction directly from deproteinized muscle extracts, the trichloroacetic acid extract from muscle was extracted with ether in order to remove the bulk of the acid, neutralized with ammonia and freed from ether by aeration, whereupon the solution was subjected to ion-exchange chromatography. The yield of nucleoside triphosphate obtained was practically identical with and in no case higher than that prepared *via* the isolated barium salts.

In the first experiments the resolution of the ATP-free nucleoside triphosphate mixture was achieved by fractional reprecipitations of the barium salts. The barium salts were treated with 0.1 *N* hydrochloric acid at 0 °C by thorough mixing with successive portions of acid for a few minutes and centrifuging after each treatment. The acid solutions were neutralized with ammonia first to pH 4.5 and then to pH 7.5, followed by centrifugations. By pooling similar fractions and repeating the procedure, UTP and GTP of over 95 % purity were ultimately obtained, as judged by paper chromatography; UTP being accumulated in the most soluble and GTP in the least soluble fractions.

For the separation of UTP and GTP by paper chromatography, the barium nucleoside triphosphate mixture was freed from barium with Dowex-50 and neutralized with ammonia. Chromatography was performed in the saturated ammonium sulphate-water-*iso*-propanol (79:19:2) solvent system on Whatman No. 1 paper after equilibration overnight, as described previously[23]. For the quantitative evaluation of the chromatograms, the spots were cut out from the paper, cut into pieces of 2 × 2 mm and extracted with 0.1 *N* hydrochloric acid for 8 hours. Optical densities were read against an extract from a paper blank of the same area cut out from beside the spot; the same blank was used for phosphorus estimations.

Separation of UTP and GTP by ion-exchange chromatography was performed on Dowex-1 formate columns, using the formate elution system, as described elsewhere[13]. UTP was isolated as the barium salt by addition of 2 *N* barium acetate solution to the pooled effluent fractions brought to pH 7 with ammonia. The barium salt was washed with water, ethanol and ether and dried *in vacuo* over phosphoric oxide. Yields of 1—4 mg pure UTP (calc. as the free acid) were obtained from 100 g muscle. (Found: P 11.0; uracil: pentose: total P: acid-labile P, 1:1:3:2. Calc. for $(C_9H_{11}O_{15}N_2P_3)Ba_2$, $4H_2O$: P: 11.3 %; uracil: pentose: total P: acid labile P 1:1:3:2).

Analytical procedures. Phosphorus was determined by the method of Allen[24] using a Klett Summerson photoelectric colorimeter with a red filter. Acid-labile phosphorus was determined after 10 minutes hydrolysis in *N* hydrochloric acid at 100 °C. Pentose estimations were carried out with the orcinol reagent according to Albaum and Umbreit[15] or before and after treatment with bromine according to Massart[16]. Optical densities were measured in a Beckman Universal Spectrophotometer Model DU with Photomultiplier.

Copper complex formation was measured by applying the procedure described by Caputto, Leloir, Cardini and Paladini[3].

Oxidation with periodate was first carried out by colorimetric estimation of the excess of periodate after reaction for 24 hours at 20° C, whereby the oxidation of *p*-phenylene diamine to a black pigment (Bandrowski's base) by periodate was used for the colori-

metric procedure. Later, the method of Dixon and Lipkin[18] was used exclusively and all previous analysis were repeated with this method.

Electrometric titration. The pure barium salt of UTP was freed from barium by Dowex-50 in the hydrogen form, and the solution of the free acid thus obtained was used for titration after analysis. 5 μmoles were titrated with 0.10 *N* sodium hydroxide in an atmosphere of nitrogen. Stirring was effected by bubbling nitrogen through the solution. A Beckman pH meter, Model G was used for the measurements.

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