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The Isolation of Guanosine-5′-Triphosphate from Muscle

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The isolation of guanosine-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 of the compound before hydrolysis. Xanthosine-5′-triphosphate was prepared by deamination of guanosine-5′-triphosphate.

The isolation of guanosine triphosphate (GTP) from rabbit skeletal muscle has been recently reported[1] from this laboratory. This, together with the isolation of uridine triphosphate (UTP) from muscle[1] and yeast[2], followed by the demonstration of cytidine triphosphate (CTP), besides GTP and UTP in various tissues[3], establishes the natural occurrence of ribonucleoside triphosphates other than adenosine triphosphate (ATP), the only previously known representative of this type of compound in nature.

It was to be expected that if, besides ATP, additional nucleoside triphosphates are present in muscle, they would be separated from other types of acid-soluble phosphorus compounds by the methods used for the isolation of ATP from deproteinized tissue extracts, appearing as contaminants in the final ATP preparation. According to expectations, the presence of a contaminating triphosphate fraction was revealed upon subjecting ATP, which had been prepared from muscle, to chromatographic purification.

Ion-exchange chromatography on the strong base anion-exchange resins Dowex-1 or Dowex-2 in the chloride form, according to Cohn and Carter[4], gave a fraction appearing in the effluent by elution with *N* hydrochloric acid, after the ATP has been removed from the column by the eluting solvent of next lowest anion content. This fraction, regularly contaminating our ATP preparations, has been subsequently recognized as a mixture of GTP and UTP. In later experiments, a further contaminating phosphate fraction was observed on ion-exchange chromatography of muscle ATP preparations. This fraction appeared in the effluent by elution with 0.01 *M* hydrochloric acid containing 0.02 *M* sodium chloride, following the adenosine diphosphate fraction and prior to the removal of ATP from the column. Phosphorus analysis indicated a tri-

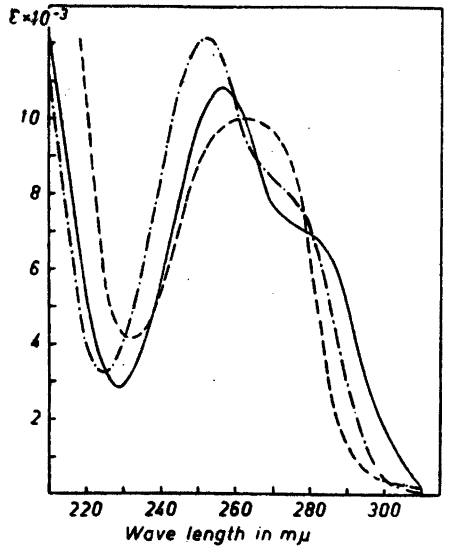


Fig. 1. Ultraviolet absorption curves for GTP from muscle. ——— *at pH 1.0,* — — — — *at pH 6.5 and* —·—·—·— *at pH 11.0.*

phosphate containing two acid-labile phosphate groups and paper chromatography after hydrolysis for one hour in *N* hydrochloric acid at 100° C gave R_F values identical with those obtained with synthetic cytidine-5′-phosphate (CMP). Ultraviolet absorption measurements on the eluted spots showed likewise identity of the product of hydrolysis with synthetic 5′-CMP. Our results indicate identity of this contaminating phosphate fraction with CTP, found in amounts corresponding to 0.2—0.3 % of the ATP, as compared to the nearly ten times larger quantities of GTP and UTP obtained from the same muscle extract. Our findings confirm the previously reported[3] occurrence of CTP in muscle and show at the same time the suitability of the barium salt precipitation for the separation of all the four ribonucleoside triphosphates from muscle extracts.

Application of the ion-exchange procedure directly to trichloroacetic acid extracts of muscle yields similar amounts of the four nucleoside triphosphates.

For the isolation of pure GTP from the GTP—UTP mixture obtained on ion-exchange chromatography, fractional reprecipitation of the barium salts and — in later experiments exclusively — ion-exchange on Dowex-1 formate columns[5] was employed, GTP being isolated as the barium salt from pooled fractions of the effluent.

Ultraviolet absorption measurements on the pure triphosphate in acid and in alkali indicate a guanosine derivative (Fig. 1) and pentose estimations with the orcinol reagent[6] show the presence of directly estimable pentose as expected for a purine nucleoside derivative. Phosphorus analysis gives values corresponding to three phosphate groups for each pentose unit in the molecule, two-thirds of the phosphorus being present in the form of acid-labile phosphate groups.

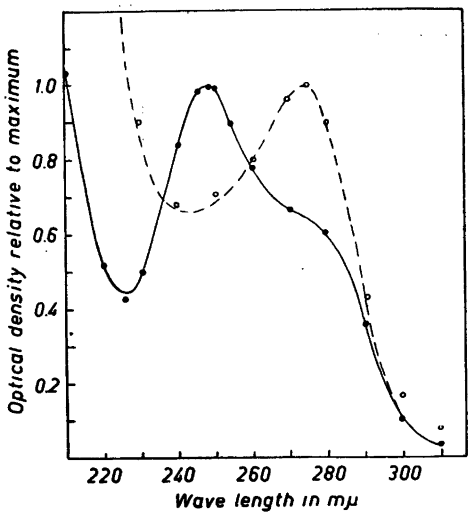


Fig. 2. Ultraviolet absorption curves for guanine obtained from GTP by hydrolysis in 70% perchloric acid or N hydrochloric acid (1 hour at 100 °C) in comparison with authentic guanine in 0.1 N acid and 0.1 N alkali. ———— guanine in acid and — — — — in alkali. ● guanine from GTP in acid and ○ in alkali.

Identification of the purine base obtained on hydrolysis of the triphosphate with 70 % perchloric acid or with *N* hydrochloric acid for one hour at 100° C, was performed by paper chromatography. R_F values identical with those of authentic guanine were obtained in four different solvent systems (Table 1). The ultraviolet absorption curves of the product of hydrolysis in acid and in alkali were likewise found to be identical with those of guanine (Fig. 2). On hydrolysis of the triphosphate with *N* sulphuric acid, guanine could be isolated as the sulphate in the form of colourless needless, indistinguishable under the microscope from the sulphate prepared from authentic guanine under identical conditions. The ultraviolet absorption curve, identical for both preparations, gave the calculated guanine content.

Table 1. R_F values of the ultraviolet absorbing base obtained from GTP (1) by hydrolysis in 70 % perchloric acid (1 hour at 100 °C) and (2) by hydrolysis in N hydrochloric acid (1 hour at 100 °C) in comparison with guanine. 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
Base (1)	0.25	0.14	0.12	0
Base (2)	0.25	0.14	0.12	0
Guanine	0.25	0.14	0.12	0

Table 2. R_F values of pentose derivatives obtained from GTP on hydrolysis with Dowex-50 (hydrogen form) for 2 hours at 100 °C, followed by descending chromatography in n-*butanol-acetic acid-water (4:1:5).*

Compound	R_F	
	Developed with aniline hydrogen phthalate	Developed with molybdate reagent
Pentose from GTP	0.32	—
D-Ribose	0.32	—
Pentose-phosphate from GTP	0.06	0.06
D-Ribose-5′-phosphate	0.06	0.06

For the identification of the pentose part of the molecule, the triphosphate was hydrolysed in *N* hydrochloric acid for one hour or with Dowex-50 resin acid for two hours at 100° C; in the last case the base formed during hydrolysis is absorbed on the resin, the solution containing the products formed from the pentose — phosphate part of the molecule. Paper chromatography revealed the presence of ribose and ribose-5′-monophosphate in the hydrolysed solution (Table 2), no other spots being obtained on development of the chromatogram with *m*-phenylene diamine or aniline hydrogen phthalate.

The identification of guanine and ribose establishes the triphosphate as a derivative of guanosine; the presence of ribose-5′-phosphate among the products of hydrolysis indicates a derivative of guanosine-5′-monophosphate (5′-GMP). In confirmation of this, 5′-GMP was obtained on heating a solution of the free triphosphoric acid for two hours to 100° C with splitting off of two-thirds of the bound phosphorus in the form of orthophosphate. Fig. 3 shows the ultraviolet absorption curves for GMP from GTP in comparison with synthetic 5′-GMP. Location of the phosphate group in the 5′-position is shown by the action of 5′-nucleotidase from snake venom[7] (Table 3).

Table 3. Action of 5′-nucleotidase on GMP obtained from GTP. 1 µmole nucleotide in 0.1 ml M *glycine buffer (pH 8.5) + 0.1 ml* 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 time of incubation		µmoles ortho-phosphate formed
	0 min.	45 min.	
Adenosine-3′-phosphate	0.005	0.005	0
Adenosine-5′-phosphate	0.01	0.92	0.91
Guanosine-3′-phosphate	0.05	0.05	0
Guanosine-5′-phosphate	0.02	0.93	0.91
Guanosine phosphate from GTP	0	0.90	0.90

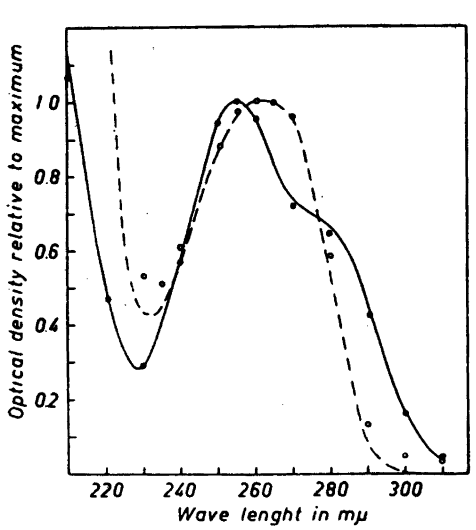


Fig. 3. Ultraviolet absorption curves for GMP obtained from GTP in comparison with synthetic 5′-GMP in 0.1 N acid and in 0.1 N alkali. ——— 5′-GMP in acid and — — — — in alkali. ● GMP from GTP in acid and ○ in alkali.

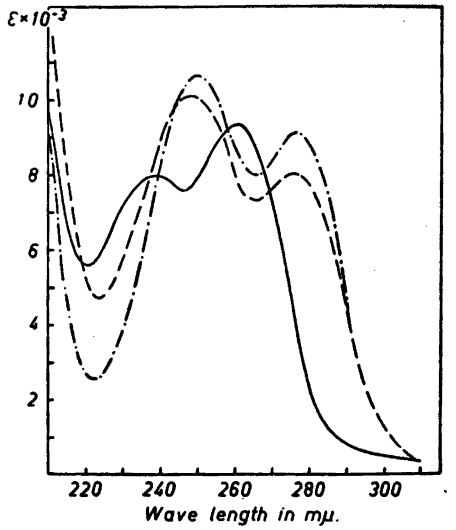


Fig. 4. Ultraviolet absorption curves for XTP, obtained by deamination of GTP. ——— at pH 1.0, — — — at pH 6.5 and —·—·—·— at pH 11.0.

As regards the attachment of the two acid-labile phosphate groups to the 5′-GMP moiety in GTP, the 2′- and 3′-positions are excluded on the basis of the results obtained in experiments on the formation of copper complex from GTP and on its oxidation with periodate. GTP forms a copper complex in analogy to 5′-substituted nucleoside derivatives[8] and in contrast to those substituted in the 2′- or 3′-positions (Table 4). The absence of substituting groups in the 2′- and 3′-positions of GTP is further demonstrated on oxidation

Table 4. Copper complex formation[9]. 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
Guanosine-3′-phosphate	62
Guanosine-5′-phosphate	315
Guanosine	335
Guanosine triphosphate	318

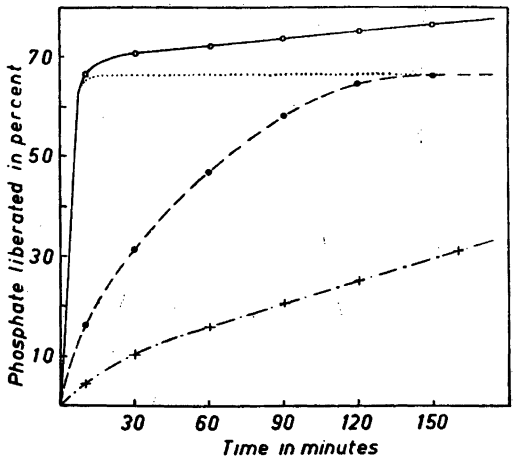


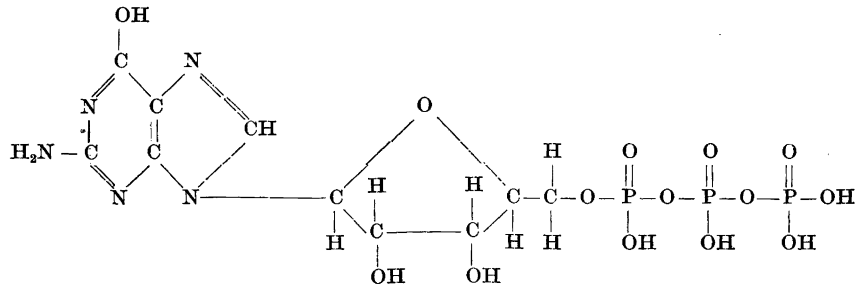
Fig. 5.
Acid hydrolysis of GTP at 100°C. ——— *in N hydrochloric acid,* – – – – *in 0.1 N hydrochloric acid and* — · — · — *synthetic 5′-GMP in N hydrochloric acid.* ····· *represents the hydrolysis of the labile phosphate groups in GTP and is obtained by subtraction of one-third of the % phosphorus liberated from 5′-GMP from the curve obtained in N acid for GTP.*

with periodate under conditions where no splitting off of the labile phosphate groups takes place (Table 5).

Deamination of GTP with nitrous acid results in the formation of xanthosine triphosphate (XTP), proving the absence of substituting phosphate groups in the amino-group of guanine in GTP. XTP was isolated as the barium salt and identified by phosphorus analysis and qualitative and quantitative evaluation of the xanthine spot obtained on paper chromatography from XTP after hydrolysis for one hour in *N* hydrochloric acid at 100° C, in comparison with authentic xanthine. The ultraviolet absorption curves for XTP at different pH values are shown in Fig. 4.

Our results, showing the presence of the 5′-GMP moiety and that of two acid-labile phosphate groups attached thereto in positions other than 2′-, 3′- and the amino-group of guanine, suggest the formulation of the GTP isolated from muscle as guanosine-5′-triphosphate.

The rates of acid hydrolysis for GTP (Fig. 5) as compared with ATP and the results of the electrometric titration (Fig. 6), showing the presence of three primary and one secondary phosphate groups in GTP, substantiate further this formulation:



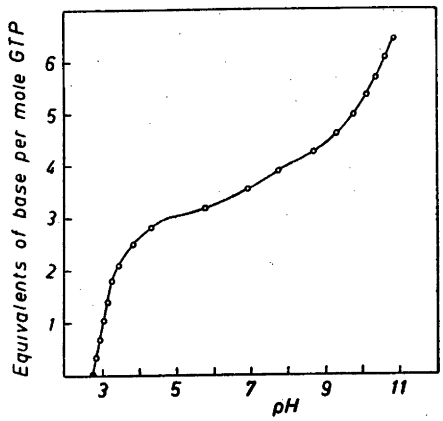


Fig. 6. Electrometric titration of GTP. 5 μmoles titrated with 0.10 N sodium hydroxide.

EXPERIMENTAL

Isolation of GTP. The preparation of barium GTP from rabbit skeletal muscle was carried out as described for UTP[11], by using as starting materials barium ATP preparations from muscle[12] or the crude mixture of barium salts obtained from the neutralized trichloroacetic acid extract of muscle in the first step of the preparation of ATP. Ion-exchange chromatography on Dowex-1 or Dowex-2 chloride columns, followed by resolution of the resulting GTP – UTP mixture by fractional precipitation of the barium salts, or, mostly, by ion-exchange on a Dowex-1 formate column in the formate system, gave pure GTP, isolated ultimately as the barium salt from pooled fractions of the effluent. In some experiments, ion-exchange chromatography in the formate system was applied directly to the starting material. Separation of GTP from UTP and ATP by paper chromatography has been described previously[11]. Yields of 1 – 4 mg of pure GTP (calc. as the free acid) were obtained from 100 g muscle; the same or a somewhat lower yield was obtained when the deproteinized muscle extract was subjected directly to ion-exchange chromatography.

The barium salt was washed with water, ethanol and ether and dried *in vacuo* over phosphoric oxide. (Found: P 10.2 %; guanine: pentose: total P: acid-labile P, 1:1:3:2. Calc. for $(C_{10}H_{12}O_{14}N_5P_3)Ba_2, 4H_2O$: P 10.7 %; guanine: pentose: acid-labile P, 1:1:3:2). Molar extinction coefficients of 10 850 at pH 1.0 (256 mμ), 12 150 at pH 6.5 (253 mμ) and 10 080 at pH 11.0 (260 mμ) were obtained (± 2 %).

Table 5. Periodate oxidation[10]. 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
Guanosine-3′-phosphate, 0.38 μmole	0.50	0.05	0.09
Guanosine-5′-phosphate, 0.37 μmole	0.50	0.36	0.98
Guanosine triphosphate, 0.36 μmole	0.50	0.35	0.97

Analytical procedures. Phosphorus was determined by the method of Allen[13], and 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[6]. 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[9].

Oxidation with periodate was performed according to Dixon and Lipkin[10].

Electrometric titration. The pure barium salt of GTP was freed from barium by Dowex-50 (hydrogen form), and the analysed solution of the free acid thus obtained was used for titration. 5 μmoles were titrated with 0.10 *N* sodium hydroxide in a nitrogen atmosphere; stirring was effected by a continuous stream of nitrogen through the solution. A Beckman pH meter, Model G was used for the measurements.

Isolation of guanine sulphate. 25 mg barium GTP in 5 ml *N* sulphuric acid were heated to 100 °C for one hour. After removal of barium sulphate by centrifugation, the solution was left overnight at 0 °C. After filtration, washing with water and drying *in vacuo* over phosphoric oxide 2.3 mg of colourless needles were obtained, indistinguishable from the product obtained from guanine (Roche) under identical conditions. (Found: guanine 69.6 %. Calc. for $(C_5H_5ON_5)_2$, H_2SO_4, 2 H_2O: guanine 69.3 % by using the molecular extinction coefficient of 10 850 at 256 mμ).

Preparation of XTP. The preparation of XTP by deamination of GTP with nitrous acid was carried out in acetate buffer of pH approximately 4 by application of the procedure used by Kleinzeller[14] for the preparation of inosine triphosphate (ITP)from ATP. To 10 μmoles sodium GTP in 0.6 ml water, 0.25 ml of a solution, prepared from 2.85 g anhydrous sodium acetate, 10 ml glacial acetic acid and 10 ml water, was added, followed by the addition of 0.1 ml 60 % sodium nitrite solution. After standing for 6 hours at 20° C the solution was cooled with ice and 2 *N* ammonia was added until the pH was approx. 7, followed by 50 μl 2 *N* barium acetate solution. The precipitate was spun off, washed with water and dissolved in 1.5 ml 0.2 *N* hydrochloric acid. After addition of 10 mg urea the solution was left for one hour at 0 °C. The barium salt was then precipitated by addition of 2 *N* ammonia until the pH was approx. 7 and then spun off. After one more reprecipitation the barium salt was washed with water and dried *in vacuo* over phosphoric oxide. Paper chromatography in the saturated ammonium sulphate solution-water-*iso*propanol (79:19:2) solvent system[15,16] gave only one ultraviolet absorbing and phosphorus containing spot, with an R_F value of 0.56 as compared with the R_F values obtained for ATP, 0.40; GTP, 0.64; ITP, 0.73 and UTP, 0.84, when chromatographed side by side on the same paper.

On hydrolysis of XTP for one hour in *N* hydrochloric acid at 100 °C, followed by ascending paper chromatography in the 5 % potassium dihydrogen phosphate-*iso*-amyl alcohol solvent system only one ultraviolet absorbing spot was observed, with the same R_F value (0.56) as given by xanthine, chromatographed side by side with the experimental solution.

Phosphorus analysis before hydrolysis in conjunction with ultraviolet absorption measurements on the eluted xanthine spot from XTP (using a molecular extinction coefficient of 9 350 at 262 mμ) gives the calculated ratio of 1:3:2 for xanthine: total phosphorus: acid-labile phosphorus.

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