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# Studies on the Occurrence and Biosynthesis of Adenosine Tetraphosphate*

Gary D. Small† and Cecil Cooper

ABSTRACT: Adenosine 5′-tetraphosphate has been detected in extracts of rabbit and horse muscle in amounts corresponding to 0.03–0.04% of the total adenosine mononucleotide content. The adenosine 5′-tetraphosphate was determined with a specific enzyme that hydrolyzes only the terminal phosphate of the nucleotide. Adenosine 5′-tetraphosphate is formed by a reaction between 1,3-diphosphoglycerate and adenosine triphosphate (ATP) catalyzed by yeast 3-phosphoglycerate kinase. The rate of this reaction is about $10^{-4}$ times the rate of the transfer of a phosphoryl group from 1,3-diphosphoglycerate to ADP catalyzed by the same enzyme. The analogous enzyme was purified from rabbit muscle and found not to catalyze this reaction. The possible significance of the small amount of adenosine 5′-tetraphosphate detected in muscle is discussed.

Adenosine 5′-tetraphosphate has been reported as a contaminant of adenosine triphosphate (ATP[1]) prepared from various biological sources (Marrian, 1954; Lieberman, 1955; Sacks, 1955), but there is no published data concerning the level of the nucleotide in tissues or its biological synthesis. The purification of an enzyme that cleaves only the terminal phosphate (Small and Cooper, 1966) provided a convenient and sensitive assay for adenosine 5′-tetraphosphate in extracts of various tissues. This paper reports the detection of adenosine 5′-tetraphosphate in rabbit and horse muscle and summarizes studies concerning the biological synthesis of the nucleotide.

## Experimental Procedures

*Measurement of Enzyme Activities.* 3-Phosphoglycerate kinase was assayed by coupling it to glyceraldehyde 3-phosphate dehydrogenase. The assay medium contained 40 μmoles of Tris, pH 7.4, 10 μmoles of cysteine or 2-mercaptoethanol, 2.5 μmoles of $MgSO_4$, 6 μmoles of 3-phosphoglycerate, 0.3 μmole of NADH, and 4 U of glyceraldehyde 3-phosphate dehydrogenase in a final volume of 1.0 ml. The reaction was started by the addition of 3-phosphoglycerate kinase and followed by measuring the decrease in optical density at 340 mμ.

Myokinase was assayed by coupling it with the pyruvate kinase–lactic dehydrogenase system. The assay medium contained 40 μmoles of Tris, pH 7.4, 15 μmoles of $MgSO_4$, 50 μmoles of KCl, 4 μmoles of phosphoenolpyruvate, 6 μmoles of ATP, 6 μmoles of AMP, 0.6 μmole of NADH, 5 U of pyruvate kinase, and 7 U of lactic dehydrogenase in a final volume of 3.0 ml. The reaction was started by the addition of myokinase and followed by measuring the decrease in optical density at 340 mμ. An enzyme unit in all cases is defined

* From the Department of Biochemistry, Western Reserve University, School of Medicine, Cleveland, Ohio. *Received August 18, 1965; revised October 8, 1965.* This work was supported by research grants (GM-5302 and GM-10446) and a training grant (5T1-GM35) from the National Institutes of Health.

† The data are from the thesis presented by G. D. Small in partial fulfillment of the requirements for the Ph.D. degree from Western Reserve University. Present address: Department of Biochemistry, University of Washington, Seattle, Wash.

[1] Abbreviations used are: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; $AP_4$, adenosine 5′-tetraphosphate; CTP, cytidine 5′-triphosphate; dATP, deoxyadenosine 5′-triphosphate; NADH, reduced nicotinamide-adenine dinucleotide.

as the amount of enzyme that catalyzes the reaction of 1 μmole of substrate/min at 25°.

*Determination of Protein.* Protein was determined by the method of Lowry *et al.* (1951) or the spectrophotometric method of Warburg and Christian (1941).

*Ion-Exchange Chromatography of Nucleotides.* Nucleotides were separated by ion-exchange chromatography on 0.8 × 11 cm columns of Dowex 1 ($Cl^-$) as described in the preceding publication (Small and Cooper, 1966).

*Deamination.* Adenosine tetraphosphate was deaminated with nitrous acid, and the product was isolated as the barium salt and converted to the sodium form as previously described (Small and Cooper, 1966).

*Detection of Adenosine 5'-Tetraphosphate in Tissue Extracts.* The method described is that used for rabbit muscle. The same general method was used for horse muscle and rabbit kidney and liver. The muscle from the hind legs of a freshly killed rabbit was quickly removed, cut into small pieces, and dropped into liquid nitrogen. The frozen muscle was broken into smaller pieces and homogenized with a volume of 0.6 N perchloric acid equal to two times the weight of the muscle. The homogenate was squeezed through a double layer of cheese cloth and the residue re-extracted with the same volume of perchloric acid. The turbid solution was filtered on a Büchner funnel. The solution was kept cold throughout these steps. The nucleotides were then concentrated by adsorption on a column of charcoal. Charcoal (60 mg, Darco G-60, untreated) was used for every 100 optical density units (optical density/cm × total ml of solution) measured at 260 mμ. The charcoal was placed on a column 5 cm in diameter that contained 3–4 cm of packed Celite 545 (untreated). After the acid extract was passed through the column it was washed with 50 ml of water. The nucleotides were then eluted with a solution composed of 1 ml of concentrated ammonium hydroxide and 100 ml of 50% ethanol, and the eluate was lyophilized to dryness. The residue was dissolved in a minimal amount of water and the following additions made: 5 μmoles of $MgSO_4$, 30 μmoles of glucose, and 1 U of hexokinase/ml of solution. The tube was incubated 30 min at 37° and the medium was then transferred to a 0.8 × 24 cm Dowex 1 ($Cl^-$) column. A maximum amount of about 600 μmoles of adenine nucleotides could be successfully separated on this column. Following the removal of ADP, the column was eluted with 0.2 N HCl–0.2 M KCl, and 3-ml fractions were collected. The fractions were immediately chilled in ice and neutralized by the addition of solid Tris. The neutralized fractions were assayed for adenosine tetraphosphate using the spectrophotometric assay previously described (Small and Cooper, 1966). About 0.1 U of nucleoside tetraphosphate hydrolase was used for each assay.

*Purification of Rabbit Muscle Myokinase.* Myokinase was partially purified by a modification of the method of Noda and Kuby (1957). The following changes were made. After the pH of fraction II was adjusted to 2, the solution was heated to 85° and maintained at that temperature for 2 min. The solution was then cooled, the pH raised to 7.2 with 5 N NaOH, and the denatured protein removed by centrifugation. This solution was used as the starting material for fraction V, and the purification was continued as described until the isolation of fraction VI. The final material had a specific activity of 63 U/mg.

*Purification of Rabbit Muscle 3-Phosphoglycerate Kinase.* The starting material for this preparation was the filtrate containing the protein not absorbed onto DEAE-cellulose in step 3 of the preparation of nucleoside tetraphosphate hydrolase (Small and Cooper, 1966).

*Batch Cellulose Phosphate.* Cellulose phosphate was suspended in 0.01 M potassium phosphate, pH 6.8, and allowed to settle overnight. A volume of the gravity-packed cellulose phosphate equal to the volume of the crude enzyme was filtered on a Büchner funnel. The cake was added to the DEAE-cellulose filtrate, stirred intermittently for 30–40 min, and filtered on a Büchner funnel, and the filtrate was discarded. The cellulose phosphate cake was suspended in 0.01 M Tris–0.005 M 2-mercaptoethanol–0.15 M KCl, pH 7.0, using the same volume as the crude enzyme. The suspension was filtered and the filtrate discarded. The enzyme was eluted by suspending the cellulose phosphate in one volume (referred to the crude enzyme) of 0.01 M Tris–0.005 M 2-mercaptoethanol–0.5 M KCl, pH 7.0, allowing the suspension to stand for 15–20 min with intermittent stirring, and then filtering. The protein was precipitated by adding 655 g of ammonium sulfate/l. of solution. After standing overnight at 0°, the precipitate was recovered by centrifuging 15 min at 37,000 × *g*. The precipitate can be stored for at least 4 months at −20° without significant loss of enzyme activity.

*Extraction with Ammonium Sulfate.* One-eighth of the protein from the preceding stage was used. The precipitate was extracted with ammonium sulfate solutions of gradually decreasing concentration. The solutions were made by dilution of an ammonium sulfate solution, pH 7.0, which was saturated at 3°. All of the solutions were made 0.001 M with respect to 2-mercaptoethanol. One milliliter of 75% saturated ammonium sulfate was added to the precipitate for every 40 mg of protein, and the suspension was stirred for 10 min. The mixture was centrifuged and the process repeated using the same volume of 70, 65, and 60%, a second time with 60%, and 55% saturated ammonium sulfate solutions. The precipitate remaining after the last extraction was dissolved in water and called the 0–55% fraction. The supernatant solutions after each centrifugation were assayed for protein and enzymatic activity. The fractions with the highest specific activity were pooled, and solid ammonium sulfate was added until the solution was 0.95 saturated. The precipitate was collected by centrifugation after standing at 0° overnight. Table I summarizes the purification steps.

There have been previous reports of the purification of this enzyme from muscle. However, the product

TABLE I: Rabbit Muscle 3-Phosphoglycerate Kinase Purification Summary.

| Fraction | Volume (ml) | Protein (total mg) | Total U | U/mg |
|---|---|---|---|---|
| 1. Crude | 1880 | 33,200 | 399,000 | 12.0 |
| 2. pH 5.4 supernatant | 1870 | 29,400 | 460,000 | 15.7 |
| 3. DEAE-cellulose supernatant | 3030 | 23,900 | 406,000 | 17.0 |
| 4. Batch cellulose phosphate | 2040 | 7,650 | 312,000 | 40.8 |
| 5. $(NH_4)_2SO_4$ extraction | | | | |
| 75% | | 25 | 505 | 20 |
| 70% | | 92 | 4580 | 49.5 |
| 65% | | 81 | 7700 | 93.5 |
| 60% | | 78 | 9900 | 128 |
| 60% (second) | | 28 | 5800 | 208 |
| 55% | | 75 | 9500 | 127 |
| 0–55% | | 597 | 5000 | 8.5 |
| | | 976 | 42,985 | |

either had a low specific activity (about 8 U/mg) (Rao and Oesper, 1961) or had a high specific activity (about 200 U/mg) but required the use of preparative electrophoresis (Czok and Bucher, 1960; Gosselin-Rey, 1963).

*Chemicals.* Adenosine 5′-tetraphosphate was obtained from the Sigma Chemical Co. and purified as previously described (Small and Cooper, 1966). [$^{32}$P]-$H_3PO_4$ was obtained from Oak Ridge National Laboratories and hydrolyzed in 1 N HCl for 90 min at 100° prior to use in order to hydrolyze any polyphosphate.

ATP labeled with $^{32}$P in both the middle and terminal positions was prepared by incubating 10 μmoles of potassium phosphate, pH 7.0, 3 mcuries of [$^{32}$P]orthophosphate, 30 μmoles of potassium succinate, pH 7.0, 20 μmoles of $MgSO_4$, 30 μmoles of AMP, 3 μmoles of ATP, 0.1 ml of 2% bovine serum albumin, and 8–10 mg of rat liver mitochondrial protein in a final volume of 2.5 ml for 25 min at room temperature with constant shaking. At the end of the incubation period, the components were transferred to a 12-ml centrifuge tube and heated 1.5 min at 100°. The denatured protein was removed by centrifugation and the supernatant solution was transferred to a Dowex 1 ($Cl^-$) column. The column was washed with 120 ml of 0.01 N HCl and 60 ml of 0.06 N HCl. The ATP was eluted from the column with 0.1 N HCl–0.2 M KCl and immediately neutralized to pH 7 with KOH.

ADP labeled with $^{32}$P in the terminal position was prepared exactly as described for ATP, except that after removing the heat-denatured protein the supernatant solution was transferred to a tube containing 10 μmoles of $MgSO_4$, 100 μmoles of glucose, and about 30–40 U of yeast hexokinase and incubated 25 min at room temperature. The reaction was terminated by heating at 100° for 1.5 min. The contents of the tube were transferred to a 1 × 10 cm Dowex 1 ($Cl^-$) column. The column was washed with 400 ml of 0.01 N HCl and the ADP was eluted with about 65 ml of 0.1 N HCl. The eluate was immediately neutralized, diluted with about 100 ml of water, and transferred to a second Dowex column of the same size. The column was washed with 150 ml of 0.01 N HCl and the ADP was eluted with 0.1 N HCl and neutralized with 2 N KOH.

Hexokinase, glucose 6-phosphate dehydrogenase, pyruvate kinase, creatine kinase, lactic dehydrogenase, yeast 3-phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase, and aldolase were obtained from California Foundation for Biochemical Research.

## Results

*Detection of Adenosine 5′-Tetraphosphate in Tissues.* The purified nucleoside tetraphosphate hydrolase provided a convenient tool for detecting adenosine tetraphosphate in tissue extracts. The details of the procedure are described in Experimental Procedures. The ultraviolet-absorbing peak that was eluted from the column after the ADP peak was assayed for adenosine tetraphosphate using the spectrophotometric assay. With this technique, 0.15 μmole of adenosine tetraphosphate was detected in 240 g wet weight of rabbit muscle. This amount corresponds to about 0.035% of the total adenosine mononucleotide content of rabbit muscle. No significant amount of the nucleotide was detected in an extract from 22 g of rabbit kidney or in an extract from 90 g of rabbit liver, but if adenosine tetraphosphate occurred in these two tissues at the same level as in muscle it would not have been detected by this method.

The adenosine tetraphosphate sold by the Sigma Chemical Co. is obtained from horse muscle. They are able to obtain 5–10 μmoles of adenosine tetraphosphate from 100 g wet weight of horse muscle, an amount corresponding to 1–2% of the total adenosine mononucleotide content (L. Berger, 1965, personal communication). Since only a fraction of this amount

was detectable in extracts of rabbit muscle, the possibility existed that the nucleotide occurred only in certain species such as horse. Freshly frozen horse muscle was provided by the Sigma Chemical Co., and 200 g was treated as described in Experimental Procedures. Only 0.22 μmole of adenosine tetraphosphate was detectable, corresponding to about 0.03% of the total adenosine mononucleotide content. About 10 μmoles of guanosine triphosphate was detected based on its position of elution from the column and the ultraviolet absorption ratios of 250/260 and 280/260 mμ. This is about the content reported by other workers (Hurlbert, 1957). Thus, it appears unlikely that substantial degradation of the nucleoside polyphosphates occurred during shipment of the muscle. The method of isolation of the nucleotides was checked by including 6 μmoles of adenosine tetraphosphate in the perchloric acid added to 200 g of rabbit muscle; about 2 μmoles was recovered. Losses probably occur because of incomplete extraction of the tissue, incomplete elution from the charcoal, and some hydrolysis, but even after taking these losses into consideration the conclusion is reached that both rabbit muscle and horse muscle contain very small amounts of the nucleotide.

The higher yield of adenosine tetraphosphate obtained from horse muscle during the commercial preparation may result from a synthesis, either chemical or enzymatic, during the isolation procedure.

*Biosynthesis of Adenosine 5'-Tetraphosphate.* Adenosine tetraphosphate may possibly be synthesized by a transphosphorylation reaction

$$X\text{–}P + ATP \rightleftarrows X + \text{adenosine tetraphosphate}$$

or perhaps by a transfer of a pyrophosphoryl group.

$$Y\text{–}PP + ADP \rightleftarrows Y + \text{adenosine tetraphosphate}$$

A search for such reactions were made using radioactive ATP, ADP, or other phosphate compounds and looking for the incorporation of $^{32}P$ into adenosine tetraphosphate.

*Test for Exchange of [$^{32}P$]Orthophosphate into Adenosine Tetraphosphate Catalyzed by Rat Liver Mitochondria.* The majority of the ATP synthesized by mammalian tissues under aerobic conditions occurs in the mitochondria, and it is possible that adenosine tetraphosphate might also be formed by the mitochondria. The incorporation of [$^{32}P$]orthophosphate into adenosine tetraphosphate was tested as follows. Tubes containing 10 μmoles of Tris, pH 7.4, 10 μmoles of potassium phosphate, pH 7.0, containing $3 \times 10^6$ cpm of $^{32}P$, 5 μmoles of adenosine tetraphosphate or 6 μmoles of ATP, 75 μmoles of KCl, and 3 mg of mitochondrial protein in a final volume of 1.0 ml were incubated for 30 min at room temperature and the reaction was terminated by heating at 100° for 3 min. The nucleotides were separated by ion-exchange chromatography on Dowex 1 ($Cl^-$). No significant amount of radioactivity was incorporated into the adenosine tetraphosphate. In control tubes with ATP instead of adenosine tetraphosphate the ATP became labeled, showing that the mitochondria were not damaged.

*Exchange of [$^{32}P$]ADP into Adenosine Tetraphosphate Catalyzed by Rat Muscle Myokinase.* Attempts to show incorporation of [$^{32}P$]ADP or [$^{32}P$]ATP into adenosine tetraphosphate catalyzed by rat liver homogenate, rat liver mitochondria, and the soluble fraction obtained after removal of the mitochondria were negative. However, using the 105,000 × *g* supernatant fraction of rat muscle homogenate a small amount of [$^{32}P$]-ADP was incorporated into adenosine tetraphosphate (Table II). The incorporation was not abolished by heating the enzyme solution at 100° for 2 min, suggesting that the reaction may be catalyzed by myokinase. To test this 40 μmoles of Tris, pH 7.4, 10 μmoles of $MgSO_4$, 20 μmoles of freshly prepared cysteine, pH 6.5, 12 μmoles of ADP containing $1.78 \times 10^6$ cpm of $^{32}P$, 14.4 μmoles of adenosine tetraphosphate, and 100 U of purified muscle myokinase in a final volume of 2.0 ml were incubated 25 min at 37°. Then 0.1 ml of 0.5 M glucose and 15 U of hexokinase were added and the incubation was continued for 10 min. This latter step was carried out to eliminate any [$^{32}P$]ATP which might trail off the Dowex columns and contaminate the

TABLE II: Exchange of [$^{32}P$]ADP into the Adenosine Tetraphosphate Fraction Catalyzed by the 105,000 × *g* Fraction of Rat Muscle Homogenate.[a]

| Experimental Conditions | Total cpm in the $AP_4$ Fraction | μmoles of $AP_4$ at End of Incubation |
|---|---|---|
| [$^{32}P$]ADP + $AP_4$, no enzyme | 50 | 4.63 |
| [$^{32}P$]ADP | 760 | 0.0 |
| [$^{32}P$]ADP + ATP | 613 | 0.0 |
| [$^{32}P$]ADP + $AP_4$ | 1487 | 0.50 |
| [$^{32}P$]ADP + $AP_4$ boiled enzyme | 1575 | 4.50 |

[a] Hind-leg muscle from a rat was homogenized in a Waring blendor in two times its weight of 0.25 M sucrose. The homogenate was centrifuged for 10 min at 12,000 × *g* and the resulting supernatant solution 30 min at 105,000 × *g*. The supernatant solution from the latter centrifugation was used as source of the enzyme. Protein (20 mg) was used per incubation tube. The incubation was at 37° for 20 min. The incubation tubes contained 40 μmoles of Tris, pH 7.4, 40 μmoles of KCl, 10 μmoles of $MgSO_4$, 6 μmoles of [$^{32}P$]ADP containing $1.3 \times 10^6$ cpm, 6 μmoles of ATP or 5 μmoles of adenosine tetraphosphate. The reaction was terminated by heating at 100° for 2 min. The denatured protein was removed by centrifugation and the supernatant solution was put on Dowex 1 columns.

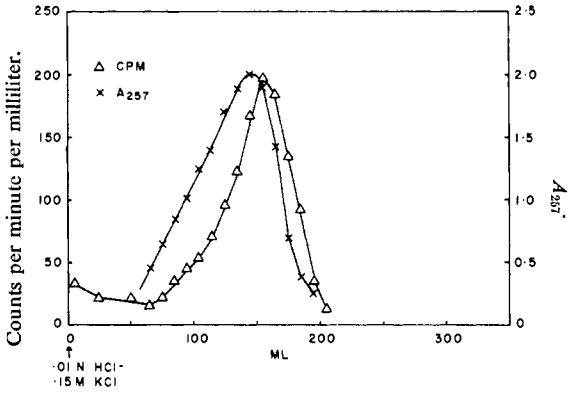



FIGURE 1: Exchange of [$^{32}$P]ADP into adenosine tetraphosphate catalyzed by myokinase.

adenosine tetraphosphate fraction. Adenosine tetraphosphate does not react with hexokinase (Liebermann, 1955). The nucleotides were separated on Dowex 1 ($Cl^-$) columns. Figure 1 shows the ultraviolet-absorbing peak and the radioactive peak that were eluted with 0.01 N HCl–0.15 M KCl. A total of 11,000 cpm was incorporated into the material eluted with this solvent. The fact that the two peaks do not coincide suggests that the radioactive component is not adenosine 5′-tetraphosphate. The peak material was pooled, about 35 μmoles of unlabeled adenosine tetraphosphate was added, and the nucleotides were adsorbed on charcoal. The nucleotides were eluted off the charcoal with about 10 ml of a solution composed of 1:2.5:6.5 concentrated ammonium hydroxide–absolute ethanol–water. The solution was evaporated to a small volume and deaminated with nitrous acid. When this was rechromatographed, no radioactive or ultraviolet-absorbing material was eluted with 0.01 N HCl–0.15 M KCl, but both radioactive and ultraviolet-absorbing materials were eluted with 0.1 N HCl–0.2 M KCl, as would be expected for inosine 5′-tetraphosphate. Once again, the ultraviolet-absorbing peak and the radioactive peak did not coincide, pointing to the fact that the material responsible for the bulk of the ultraviolet absorption and the radioactive material are two different materials. Their similar behavior on the Dowex columns both before and after deamination suggests that the two compounds are closely related. One possibility is that the radioactive component is 3′-phosphoadenosine 5′-triphosphate, but further work is necessary to establish this point.

These studies were not pursued since it appeared that this reaction had nothing to do with the biological synthesis of adenosine 5′-tetraphosphate. The fact that net formation of this unknown radioactive compound did not occur in the absence of added unlabeled adenosine tetraphosphate and that large amounts of enzyme were required to show much incorporation of radioactivity into the unknown compound suggests that it is probably a minor side reaction catalyzed by myokinase.

*Synthesis of Adenosine Tetraphosphate Catalyzed by 3-Phosphoglycerate Kinase.* In skeletal muscle, ATP is formed from ADP by oxidative phosphorylation and by transphosphorylation reactions catalyzed by myokinase, pyruvate kinase, creatine kinase, and 3-phosphoglycerate kinase. A possible mechanism for adenosine tetraphosphate formation is the substitution of ATP for ADP as the phosphoryl acceptor as illustrated in reactions 1–4.

$$ADP + ATP \longrightarrow AP_4 + AMP \quad (1)$$

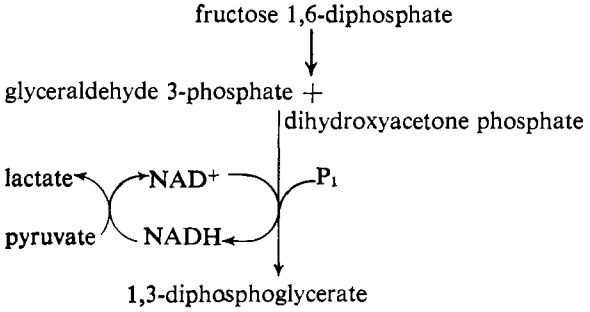
$$\text{phosphoenolpyruvate} + ATP \longrightarrow AP_4 + \text{pyruvate} \quad (2)$$

$$\text{creatine phosphate} + ATP \longrightarrow AP_4 + \text{creatine} \quad (3)$$

$$\text{1,3-diphosphoglycerate} + ATP \longrightarrow AP_4 + \text{3-phosphoglycerate} \quad (4)$$

The results in the previous section appear to rule out net formation of adenosine tetraphosphate by reaction 1 catalyzed by myokinase. Attempts to show synthesis of adenosine tetraphosphate by reactions 2 and 3 catalyzed by pyruvate kinase and creatine kinase, respectively, were also negative. The latter experiments were carried out as follows. Tubes in which pyruvate kinase was added contained 40 μmoles of Tris, pH 7.4, 10 μmoles of KCl, 10 μmoles of $MgSO_4$, 40 μmoles of phosphoenolpyruvate, 12 μmoles of [$^{32}$P]ATP containing $1 \times 10^6$ cpm, and 17 U of pyruvate kinase in a final volume of 2.0 ml. Tubes in which creatine kinase was added contained 40 μmoles of Tris, pH 7.4, 10 μmoles of $MgSO_4$, 20 μmoles of creatine phosphate, 12 μmoles of [$^{32}$P]ATP containing $1 \times 10^6$ cpm, and 28 U of creatine kinase in a final volume of 2.0 ml. The tubes were incubated 2 hr at 37°. The reaction was terminated by heating at 100° for 2.5 min. After cooling, 50 μmoles of glucose and 30 U of hexokinase were added, and the tubes were incubated 15 min at 37°. The reaction was terminated as before and the nucleotides were separated on Dowex 1 ($Cl^-$) columns. No radioactive or ultraviolet-absorbing material was eluted in the adenosine tetraphosphate fraction.

The enzyme, 3-phosphoglycerate kinase from yeast, was shown to catalyze reaction 4 at a very slow rate. 1,3-Diphosphoglycerate was generated during the reaction by the series of enzyme-catalyzed reactions outlined below. The incubation tubes contained 40 μmoles of Tris, pH 7.4, 10 μmoles of $MgSO_4$, 12 μmoles of



[$^{32}$P]ATP containing 1 × $10^6$ cpm, 40 μmoles of fructose 1,6-diphosphate, 30 μmoles of pyruvate, 40 μmoles of cysteine, pH 7.4, 3 μmoles of $NAD^+$, 12 U of aldolase, 13 U of glyceraldehyde 3-phosphate dehydrogenase, 15 U of lactic dehydrogenase, and 50 U of yeast 3-phosphoglycerate kinase. Incubation conditions and separation of the nucleotides was exactly as described above. Figure 2 illustrates that a radioactive and ultraviolet-absorbing substance is eluted from the column with 0.01 N HCl–0.15 M KCl exactly at the place where adenosine tetraphosphate is eluted. The spectrum of this fraction is indistinguishable from that of commercial adenosine tetraphosphate at pH 2 and 7.4.

The peak fractions were combined, 25 μmoles of unlabeled adenosine tetraphosphate was added, and the nucleotide was adsorbed on 100 mg of charcoal (Norit A, boiled in 1 N HCl and washed with water). The nucleotide was eluted with about 10 ml of 1:2.5:6.6 concentrated ammonium hydroxide–absolute ethanol–water, evaporated to a small volume, and deaminated with nitrous acid. When this deaminated material was chromatographed on a Dowex 1 column both the radioactivity and optical density coincided and were eluted in the inosine tetraphosphate region. This is in contrast to the results obtained with myokinase.

Table III summarizes an experiment showing that

TABLE III: Synthesis of Adenosine Tetraphosphate Catalyzed by Yeast 3-Phosphoglycerate Kinase.[a]

| Experimental Conditions | Total cpm in $AP_4$ Fraction | μmoles $AP_4$ Formed |
|---|---|---|
| Complete, zero time | 0 | 0.0 |
| Complete | 126,300 | 0.84 |
| − Fructose 1,6-diphosphate | 0 | 0.0 |
| − NAD | 0 | 0.0 |
| − Pyruvate + lactic dehydrogenase | 10,140 | 0.19 |
| − 3-Phosphoglycerate kinase | 0 | 0.0 |
| − Aldolase | 18,770 | 0.27 |
| − Glyceraldehyde 3-phosphate dehydrogenase | 12,580 | 0.40 |

[a] The complete system contained 40 μmoles of Tris, pH 7.4, 10 μmoles of $MgSO_4$, 12 μmoles of ATP, 20 μmoles of [$^{32}$P]$K_3PO_4$, pH 7.4, containing 3.2 × $10^6$ cpm, 40 μmoles of fructose 6-diphosphate, pH 7.0, 3 μmoles of NAD, 30 μmoles of pyruvate, 15 U of lactic dehydrogenase, 40 μmoles of cysteine, pH 7.4, 12 U of aldolase, 13 U of glyceraldehyde 3-phosphate dehydrogenase, and 50 U of yeast 3-phosphoglycerate kinase in a final volume of 2.0 ml. The tubes were incubated for 2 hr at 37°. The reaction was terminated by heating at 100° for 2.5 min; 15 U of hexokinase and 25 μmoles of glucose were added and the tubes incubated 15 min at 37°.

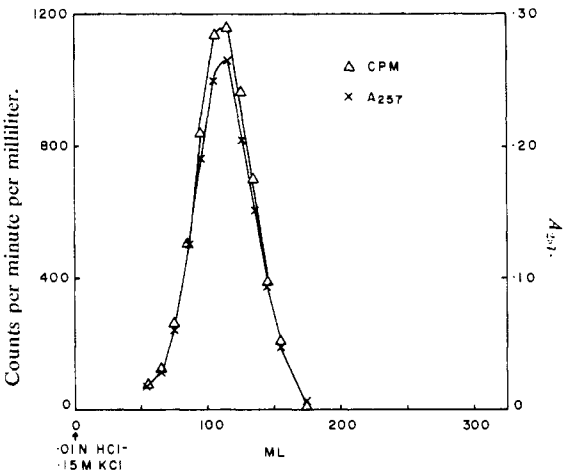



FIGURE 2: Synthesis of adenosine tetraphosphate catalyzed by yeast 3-phosphoglycerate kinase.

all components are necessary for maximum synthesis of adenosine tetraphosphate. The small amount formed when the enzymes aldolase and glyceraldehyde 3-phosphate dehydrogenase are omitted is probably attributable in the presence of small amounts of these enzymes as contaminants of the other enzymes added. The experiment also shows that [$^{32}$P]orthophosphate can be incorporated into adenosine tetraphosphate, as would be predicted.

Synthesis of adenosine tetraphosphate catalyzed by yeast 3-phosphoglycerate kinase can be shown using a different, and less complex, method of generating relatively high levels of the phosphoryl donor, 1,3-diphosphoglyceric acid. This is illustrated by reactions 5–7.

$$\text{ATP} + \text{3-phosphoglycerate} \longrightarrow \text{1,3-diphosphoglycerate} + \text{ADP} \quad (5)$$

$$\text{ADP} + \begin{matrix}\text{phosphoenolpyruvate} \\ \text{or} \\ \text{creatine phosphate}\end{matrix} \longrightarrow \begin{matrix}\text{pyruvate} + \text{ATP} \\ \text{or} \\ \text{creatine}\end{matrix} \quad (6)$$

$$\text{ATP} + \text{1,3-diphosphoglycerate} \longrightarrow \text{3-phosphoglycerate} + AP_4 \quad (7)$$

The 1,3-diphosphoglycerate is formed by the reaction between ATP and 3-phosphoglycerate as shown in reaction 5. However, the equilibrium of this reaction favors the formation of ATP. By rephosphorylating the ADP back to ATP with phosphoenolpyruvate or creatine phosphate, much larger amounts of 1,3-diphosphoglycerate can be formed. Reaction 7 may now proceed, resulting in the formation of adenosine tetraphosphate. Table IV shows the results of such an experiment. Figure 3 illustrates that the formation of adenosine tetraphosphate is linear during a 2-hr in-

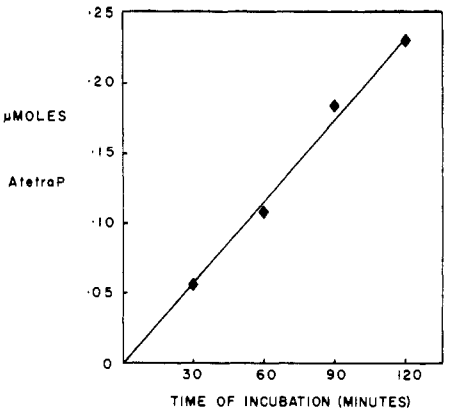



FIGURE 3: Formation of adenosine tetraphosphate in the presence of yeast 3-phosphoglycerate kinase plus an ATP-regenerating system. The incubation tubes contained 40 μmoles of Tris, pH 7.4, 10 μmoles of $MgSO_4$, 15 μmoles of 3-phosphoglycerate, 15 μmoles of ATP, 20 μmoles of creatine phosphate, 3.2 U of creatine kinase, and 24 U of yeast 3-phosphoglycerate kinase in a final volume of 2.0 ml. The reaction was terminated by heating at 100° for 3 min. The nucleotides were separated on a Dowex 1 ($Cl^-$) column.

cubation using creatine phosphate plus creatine kinase as an ATP-regenerating system.

The rate of this reaction is extremely slow compared to the transfer of a phosphoryl group from 1,3-diphosphoglycerate to ADP catalyzed by this same enzyme. The relative rates of the two reactions can be calculated to be in the neighborhood of 1:10,000. If this reaction accounts for adenosine tetraphosphate synthesis in muscle, it should be possible to carry out a similar experiment using rabbit muscle 3-phosphoglycerate kinase. The incubation was set up exactly as described for Table II using the partially purified muscle enzyme. Phosphoenolpyruvate and pyruvate kinase were used as an ATP-regenerating system. Thirty-two units of the partially purified rabbit muscle 3-phosphoglycerate kinase was used per incubation. No adenosine tetraphosphate was formed even after 2-hr incubation at 37°. Control experiments proved that the 3-phosphoglycerate kinase lost less than 10% of its activity at the end of the 2-hr incubation, and added adenosine tetraphosphate could be completely recovered after a 2-hr incubation in the presence of the kinase. Therefore, it appears unlikely that the adenosine tetraphosphate in muscle is formed in this manner.

## Discussion

The only known mechanism for the biological synthesis of adenosine tetraphosphate is the reaction between 1,3-diphosphoglycerate and ATP catalyzed by yeast 3-phosphoglycerate kinase. The finding that the analogous enzyme from rabbit muscle will not catalyze this reaction makes it unlikely that this is the mechanism

TABLE IV: Synthesis of Adenosine Tetraphosphate in the Presence of 3-Phosphoglycerate Kinase plus an ATP Regenerating System.[a]

| Experimental Conditions | AMP | ADP | ATP | $AP_4$ |
|---|---|---|---|---|
| Creatine kinase, zero time | 0.18 | 0.44 | 11.42 | 0.0 |
| Creatine kinase, 2-hr incubation | 0.09 | 0.42 | 10.95 | 0.45 |
| Pyruvate kinase, zero time | 0.12 | 0.62 | 11.29 | 0.0 |
| Pyruvate kinase, 2-hr incubation | 0.07 | 0.42 | 10.99 | 0.48 |

[a] All of the tubes contained 40 μmoles of Tris, pH 7.4, 10 μmoles of $MgSO_4$, 50 μmoles of KCl, 20 μmoles of 3-phosphoglycerate, 12 μmoles of ATP, and 21 U of yeast 3-phosphoglycerate kinase. Tubes 1 and 2 contained 20 μmoles of creatine phosphate and 10 U of creatine kinase. Tubes 3 and 4 contained 12 μmoles of phosphoenolpyruvate and 4 U of pyruvate kinase. The final volume in all cases was 2.0 ml. Incubation was at 37°. The reaction was terminated by heating at 100° for 2.5 min. The amounts of the various nucleotides were calculated from their absorption at 260 mμ using a millimolar extinction coefficient of 15.4 for all of the nucleotides.

for the formation of the small amount of adenosine tetraphosphate found in muscle. This is not the first observed difference between the yeast and rabbit muscle enzymes. The two enzymes also show a difference in nucleotide specificity. The reaction of UTP with 3-phosphoglycerate proceeds at about 10% of the rate of ATP with the yeast enzyme, whereas with the muscle enzyme the rate with UTP is less than 0.01% the rate with ATP (G. D. Small and C. Cooper, 1965, unpublished data). Also, the muscle enzyme is inhibited by *p*-mercuribenzoate (hydroxy) whereas the yeast enzyme is not (Rao and Oesper, 1961).

Approximately 0.2 μmole of adenosine tetraphosphate was detected in an extract of 200 g wet weight of horse muscle. An experiment in which a known amount of adenosine tetraphosphate was added prior to carrying out the extraction procedure resulted in the recovery of only about one-third of the added nucleotide. Thus, perhaps as much as 0.6 μmole of adenosine tetraphosphate was originally present in the 200 g of muscle, or about 3 μmoles/kg. Other nucleotides having known physiological functions occur in tissues in amounts comparable to adenosine tetraphosphate. For example, CTP occurs in rabbit muscle in amounts ranging from 6 to 12 μmoles/kg wet weight (Hurlbert, 1957). About 4 μmoles of dATP has been isolated per

kg of Flexner-Jobling carcinoma (LePage, 1957). Cyclic 3′,5′-AMP occurs in skeletal muscle at levels of 0.6–1.8 μmoles/kg (Posner *et al.*, 1965).

One possibility that must be considered is that adenosine tetraphosphate is formed during the isolation procedure. The slow nonenzymatic formation of adenosine tetraphosphate can be shown with high levels of ATP and calcium at pH 8.5 at 40° (C. Cooper, unpublished data). However, it seems unlikely that this could have occurred to a significant extent during the isolation procedure.

Another possibility is that adenosine tetraphosphate is a breakdown product of a more complex molecule. An example that one might consider is a diadenosine tetraphosphate analogous to the diguanosine tetraphosphate that has been isolated from shrimp brine eggs (Finamore and Warner, 1963).

The physiological function of adenosine tetraphosphate is completely unknown. It cannot substitute for ATP in any reaction that has been tried, but it is possible that adenosine tetraphosphate serves as a specific phosphorylating agent in some special reaction. The small amount found in muscle would appear to eliminate the nucleotide as a storage form of energy analogous to creatine phosphate. Perhaps adenosine tetraphosphate regulates certain enzymatic reactions in a manner analogous to cyclic 3′,5′-AMP.

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