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Purification and Properties of an Acid Nucleoside Triphosphatase from Rat Liver Mitochondria*

George F. Kalf† and Mary Ann Gréce

ABSTRACT: A nucleoside triphosphatase with an optimal pH of 5.5 has been partially purified from rat liver mitochondria. The enzyme shows phosphohydrolytic activity toward ribo- and deoxyribonucleoside di- and triphosphates and *p*-nitrophenyl phosphate but does not hydrolyze 5′-nucleoside monophosphates or other monophosphate esters. Activity is inhibited by low levels (1 mM) of Ca^{2+}, Mg^{2+}, and Fe^{3+} and a number of other cations but is stimulated by Fe^{2+}. The activity is virtually unaffected by organic compounds such as EDTA and L(+)-tartrate.

2-Mercaptoethanol and other sulfhydryl compounds stimulate activity. Fluoride is a potent inhibitor. Evidence obtained from column chromatography, polyacrylamide disc gel electrophoresis, and a heat study suggests that the broad substrate specificity results from the action of a single enzyme.

In this paper, we report on the purification and properties of a soluble nucleoside triphosphatase from rat liver mitochondria which has a pH optimum of 5.5. Nucleoside triphosphatases with somewhat similar properties have been reported to be present in normal and regenerating rat liver nuclei (Fischer *et al.*, 1959; Siebert and Humphrey, 1965) and pea leaves (Forti *et al.*, 1962), and an acid nucleotidase has also been purified from rat liver lysosomes (Arsenis and Touster, 1968). It is also of considerable interest that chloroplasts of *Euglena gracilis* contain a Mg^{2+}-dependent ATPase with a pH optimum of 5.5, which hydrolyzes dATP more rapidly than ATP and shows activity on other nucleoside triphosphates (Carell and Kahn, 1967). Spinach chloroplasts, on the other hand, contain a similar enzyme which does not require Mg^{2+} (Young and Packer, 1966).

Experimental Procedure

Materials. Nucleotides were supplied by either Sigma or Schwarz BioResearch. Glucose 6-phosphate, glucose 1-phosphate, α- and β-glycerol phosphate, D-3-phosphoglyceric acid, phosphoenol pyruvate, NAD, NADP, *p*-nitrophenyl phos-

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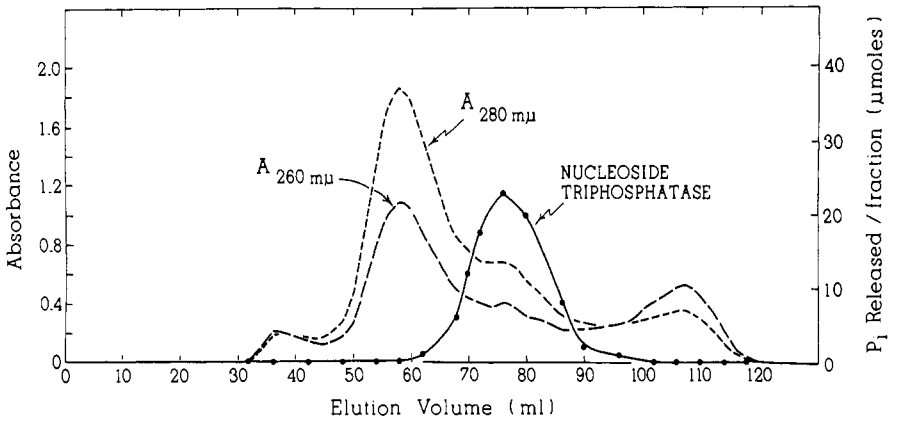

FIGURE 1: Sephadex G-200 column chromatography. A column, 1.5 × 76 cm, was prepared from Sephadex G-200 previously equilibrated with 0.6 M KCl–2 mM Tris, pH 7.4. The column was charged with 5 ml (70 mg of protein) of the ammonium sulfate fraction (50–75%) and eluted with the equilibrating buffer at a flow rate of approximately 8 ml/hr. Fractions (2 ml) were collected. Absorbance at 280 and 260 mμ was measured for each fraction and ATPase activity was determined as described in Methods: (- - - - - - - - -) $A_{280\ m\mu}$; (– – – – –) $A_{260\ m\mu}$; (●—●—●) ATPase activity.

phate, and bis-*p*-nitrophenyl phosphate were also purchased from Sigma. Casein (Hammarsten) was obtained from Nutritional Biochemicals and was prepared for use as described (Revel and Racker, 1960). Phosvitin was a gift from Dr. George Taborsky. Distillation Products was the source of 2-mercaptoethanol. *N*-Ethylmaleimide and *p*-chloromercuribenzoate were obtained from Nutritional Biochemicals. Reagents for gel electrophoresis were supplied by Fisher. DEAE-cellulose was purchased from Carl Schleicher and Schuell Co. and was regenerated before use (Peterson and Sober, 1956). Sephadex dextran gels were obtained from Pharmacia Fine Chemicals, Inc. Digitonin was purchased from Sigma and was recrystallized from absolute alcohol before use.

Methods. PREPARATION OF MITOCHONDRIAL EXTRACT. Female Wistar rats (150–170 g) were fasted 18 hr before they were decapitated. The livers were quickly removed and homogenized in ice-cold 0.34 M sucrose–2 mM Tris-HCl, pH 7.4. All subsequent operations were carried out at 0–4°. Five-times washed mitochondria were prepared as previously described by O'Brien and Kalf (1967). The yield of mitochondrial protein was 13 mg/g of liver; the purity of these mitochondria with respect to contamination by such subcellular components as nuclei, nuclear fragments, and microsomes has been described in detail (O'Brien and Kalf, 1967), and the problem of bacterial contamination has also been investigated (Kalf and Ch'ih, 1968). Such mitochondrial preparations have been shown to be virtually free of contamination by other subcellular components and bacteria. However, to further minimize the possibility of contamination of the washed mitochondria by cytoplasmic enzymes, possibly adsorbed to the outer membrane of the organelle, a separation of the outer and inner membranes of the mitochondria was achieved by the digitonin method of Schnaitman *et al.* (1967, 1968). Digitonin treatment as employed in this method does not markedly affect the integrity of the inner mitochondrial membrane and hence considerable amounts of soluble matrix proteins are retained within the isolated inner membrane (Schnaitman and Greenawalt, 1968). This procedure yielded preparations of inner membranes which were virtually free of contamination by outer membranes (5% or less) based on assay of monoamine oxidase as the outer membrane marker (Schnaitman *et al.*, 1967). Considerable amounts of matrix material were present based on assay of malate dehydrogenase.

The inner membrane–matrix fraction was suspended in cold 0.6 M KCl–2 mM Tris, pH 7.5, to a final protein concentration of 12 mg/ml, based on the amount of whole mitochondrial protein used to prepare the membranes. The suspension was homogenized and extraction was carried out with stirring for 16 hr; the suspension was then centrifuged for 30 min at 39,000 rpm in the Spinco No. 40 rotor. The extract generally contained 8–10% of the protein of the inner membrane–matrix preparation.

Ammonium Sulfate Fractionation. The KCl extract was made 1 mM with respect to 2-mercaptoethanol and Fe^{2+}. It has been observed that considerable loss of enzyme activity occurs when this fractionation is carried out in the absence of these factors and that they have both a protective and stimulatory effect on the enzyme activity.

Solid ammonium sulfate was gradually added with stirring to 0.5 saturation; the pH was maintained at 7.0 by the dropwise addition of 0.3 M ammonium hydroxide. After standing for 20 min, the precipitate was removed by centrifugation and additional ammonium sulfate was added to bring the supernatant fluid to 0.75 saturation. After 20 min, the precipitate was collected and dissolved in a minimum volume of 0.6 M KCl–2 mM Tris, pH 7.5.

Chromatography on Sephadex G-200. A column (1.5 × 76 cm) of Sephadex G-200, equilibrated with 0.6 M KCl–2 mM Tris, pH 7.4, was charged with the 50–75% ammonium sulfate fraction. The sample volume was 4% of the total column volume. Elution was accomplished with 0.6 M KCl–2 mM Tris, pH 7.4, at a flow rate of 8 ml/hr and 2-ml fractions were collected. Figure 1 presents a representative separation of nucleoside triphosphatase on Sephadex G-200. Fractions eluting between 70 and 90 ml of buffer contained the nucleoside triphosphatase activity and were combined, concentrated by passage through a Diaflo PM-10 membrane (Amicon Corp., Lexington, Mass.), and desalted on a column (1.0 × 22 cm) of Sephadex G-25 equilibrated with 2 mM Tris, pH 7.5.

Chromatography on DEAE-Cellulose. The enzyme solution

obtained from the Sephadex G-25 column was chromatographed on a column of DEAE-cellulose (1.2 × 24 cm) equilibrated with 20 mM Tris, pH 8.0. The flow rate was held constant at 40 ml/hr by means of a Buchler polystaltic pump. The eluates passed through a 10-mm quartz cell of a TMC Vanguard 1056 A ultraviolet analyzer, and the absorbance at 278 mμ was recorded. Fractions of 5 ml were collected.

Three separate peaks were eluted from the column with the equilibrating buffer, but no nucleoside triphosphatase was present in the fractions comprising these peaks. Elution of the enzyme as a single peak was achieved with 0.1 M KCl–20 mM Tris, pH 8.0. Figure 2 presents a typical profile achieved by eluting the nucleoside triphosphatase from the DEAE column with this buffer. The pH of the fractions containing the nucleotidase was immediately adjusted to 7 to increase the stability of the enzyme. Two additional peaks which did not contain nucleotidase activity were subsequently eluted at KCl concentrations of 0.17 and 0.30 M, respectively.

Enzyme Assays. The standard enzyme assay mixture contained, in a total volume of 1 ml, 5 μmoles of substrate, 50 μmoles of acetate buffer, pH 5.5, 0.4 μmole of 2-mercaptoethanol, and an amount of KCl extract or enzyme fraction which hydrolyzed less than 20% of the substrate. After 30 min at 37°, the reaction was stopped by the addition of an equal volume of 5% perchloric acid, the tubes were chilled to 0°, and the precipitated protein was removed by centrifugation. The inorganic phosphate released was measured by the method of Fiske and SubbaRow (1925).

Assay for succinic dehydrogenase was carried out as described (Bonner, 1955); malate dehydrogenase was assayed according to the method of Mehler *et al.* (1948), and monoamine oxidase by the method of Tabor *et al.* (1954).

Protein Concentration. Protein was estimated by the biuret method (Robinson and Hogden, 1940) with bovine serum albumin as a standard, or spectrophotometrically in a Zeiss PMQ II spectrophotometer by measuring the absorption of light at 280 and 260 mμ.

Polyacrylamide Gel Electrophoresis. Basic disc electrophoresis was performed in 7.5% polyacrylamide gel as described (Davis, 1964). The electrode buffer was 0.025 M Tris–0.19 M glycine, pH 8.9. Electrophoresis at acid pH was performed in 10% polyacrylamide gel, using an electrode buffer consisting of β-alanine–acetic acid, pH 4.5 (Reisfeld *et al.*, 1962). Electrophoresis was carried out with a constant current of 5 mA/tube for 1 hr in the case of the basic gel and 7 mA/tube for 3 hr with the acid gels. Protein bands were visualized by staining with 0.1% Amido black in 7% acetic acid for 1 hr, followed by electrolytic destaining. In order to detect ATPase activity on the gel, the gel was incubated with ATP under the usual enzyme assay conditions in a total volume of 10 ml. At the end of the incubation, the media was poured off and the gel was rinsed several times with water. Areas of P_i deposition on the gel were determined by the addition of the reducing mixture (Fiske, SubbaRow) to the rube. Within a few minutes a blue band appeared denoting the location of the enzyme; eventually the entire gel became blue.

Results

Purification of the Mitochondria. In order to ascertain that the nucleoside triphosphatase activity in the mitochondrial preparation was a true mitochondrial activity and not the result of contamination by cytoplasmic components, the mitochondria were repeatedly washed by resuspension in 0.34 M sucrose–2 mM Tris, pH 7.4, followed by centrifugation at 3700*g* for 10 min. The ATPase activity at pH 5.5 in both crude and washed mitochondria was determined as was the activity of succinic dehydrogenase which was used as a mitochondrial marker enzyme. The results, presented in Table I, show that after the first washing, the ratio of ATPase activity of the mitochondria to the succinic dehydrogenase activity approached a constant value and did not change significantly upon further washing. A similar situation was noted with the dATPase activity. These results suggest that the nucleoside triphosphatase activity is of mitochondrial origin. Further evidence was obtained by assaying the particle-free extract of purified inner membrane–matrix preparations for activity of a number of cytoplasmic marker enzymes. This KCl

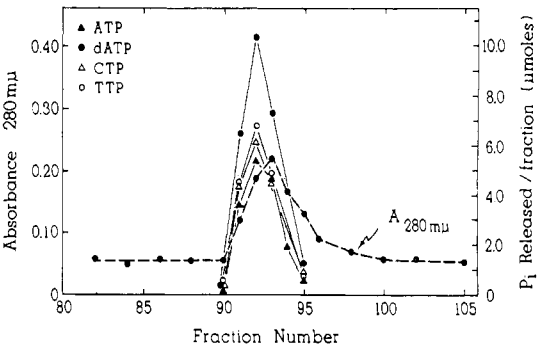

FIGURE 2: DEAE column chromatography of partially purified nucleoside triphosphatase. A column, 1.2 × 24 cm, was equilibrated with 20 mM Tris, pH 8.0, and charged with 10 ml (2 mg of protein) of an ammonium sulfate fraction (50–75%) which had previously been desalted on Sephadex G-25. The figure shows the profile of the peak containing the nucleoside triphosphatase which was eluted with 0.1 M KCl–20 mM Tris, pH 8.0. Fractions (5 ml) were collected and absorbance at 280 mμ was measured for each fraction. The activity on several representative substrates was determined: (▲———▲) ATP; (●———●) dATP; (△———△) CTP; (○———○) TTP.

TABLE I: Effect of Repeated Washing on the Nucleoside Triphosphatase Activity of Rat Liver Mitochondria.[a]

Treatment	ATPase[b] SDH[c]	dATPase[b] SDH[c]
Unwashed	0.57	0.43
Washed once	0.58	0.36
Washed twice	0.48	0.32
Washed three times	0.43	0.30
Washed four times	0.52	0.26
Washed five times	0.40	0.25

[a] The mitochondrial washed procedure and the conditions for nucleoside triphosphatase and succinic dehydrogenase assays are described in Methods. [b] ATPase or dATPase expressed as μmoles of P_i/mg of protein per 15 min. [c] Succinic dehydrogenase (SDH) expressed as ΔA_{400}/mg of protein per 20 min.

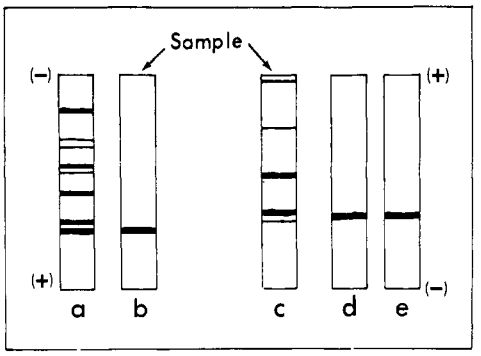

FIGURE 3: Polyacrylamide gel electrophoresis of crude mitochondrial acid nucleoside triphosphatase and of the purified enzyme. The disc electrophoretic profiles shown are (a) KCl extract of mitochondria at pH 8.9, (b) purified nucleoside triphosphatase at pH 8.9, (c) KCl extract at pH 4.5, (d) KCl extract at pH 4.5, incubated and stained for inorganic phosphate, and (e) purified nucleoside triphosphatase at pH 4.5, incubated and stained for inorganic phosphate. Enzyme protein (100 μg) was subjected to electrophoresis under conditions described in Methods. Gels were incubated for ATPase activity and stained for P_i as described in Methods.

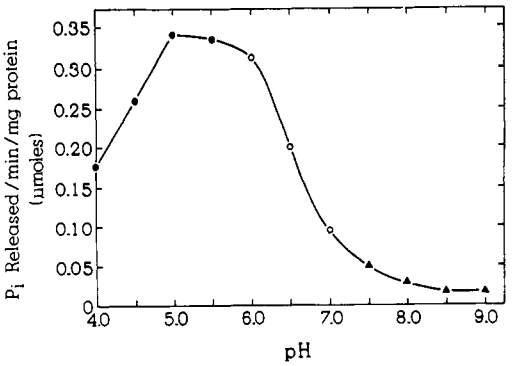

FIGURE 4: Effect of pH on the activity of the mitochondrial acid nucleoside triphosphatase. The enzyme assay and inorganic phosphate determination were carried out as described under Methods. (●——●) acetate buffer; (○——○) histidine buffer; (▲——▲) Tris acetate buffer.

extract showed virtually no activity toward α- or β-glycerol phosphate, phosphoenol pyruvate, ribo- and deoxyribonucleoside monophosphates, glucose 6-phosphate, glucose 1-phosphate, sodium pyrophosphate, D-3-phosphoglyceric acid, NAD, NADP, casein, phosvitin, or bis-*p*-nitrophenyl phosphate. Lack of activity on these substrates also suggests the absence of contamination by both microsomal and lysosomal enzymes.

Partial Purification of the Acid Nucleoside Triphosphatase. It is difficult to assess the level of final purification of the enzyme because of its instability at several steps during the purification. Increases in specific activity as high as tenfold have been observed through the DEAE column. A typical purification is presented in Table II. Adsorption and elution from the DEAE column at pH 8.0 result in a considerable loss of enzyme units. Neither the addition of Fe^{2+}, ascorbic acid, nor mercaptoethanol to the active fraction restores the enzyme activity. Exposure of the enzyme to pH 8 at 4° causes a slow but irreversible loss of enzyme activity whereas exposure to pH 9 results in a rapid and complete loss of activity. For this reason, elution of the enzyme from the column was accomplished as rapidly as possible with the stepwise gradient and the pH of the fractions containing the enzyme was immediately adjusted to 7.0. However, some purification is achieved in the column step as evidenced by the studies with polyacrylamide gels described below. The activity of the DEAE-purified enzyme deteriorates rapidly; however, KCl extracts and ammonium sulfate fractions maintained at −20° for as long as 1 year have shown no appreciable loss in nucleoside triphosphatase activity. Although the activity of the partially purified enzyme appears to be low, it is similar to that of other nucleoside triphosphatases as reported in the introduction (Forti *et al.*, 1962; Carell and Kahn, 1967; Young and Packer, 1966).

TABLE II: Purification of Nucleoside Triphosphatase.

Procedure	Enzyme Units[a]	Protein (mg)	Specific Activity[b]	Recovery (%)
Extract	287	224	1.3	
Ammonium sulfate	204	85	2.4	71
Sephadex G-200	116	29	4.0	40
DEAE-cellulose				
Pooled fractions	13	5	2.7	4.5
Peak tube	2.2	0.22	9.8	

[a] The substrate was ATP, the assay being carried out at pH 5.5 as described in the text. [b] The specific activity is μmoles of substrate hydrolyzed per hour per mg of protein.

Because of the requirement for a pH of 8 in order to adsorb the enzyme to DEAE-cellulose and because of its instability at this pH, attempts were made to further purify the ammonium sulfate and/or Sephadex G-200 fraction by alternative chromatographic methods. It was found that the enzyme was adsorbed to CM-cellulose at pH 4.7, but that it could not be eluted from this resin by a variety of conditions of pH and salt concentration; elution could only be accomplished with dilute NaOH and with complete loss of enzyme activity.

Electrophoresis of the KCl extract on polyacrylamide gels at pH 4.5 and 4° had indicated a separation of the components of the extract with the nucleoside triphosphatase activity localized in a single band which retained enzyme activity when assayed on the gel. This prompted the use of gel electrophoresis as a means of purification. Here, again, we were unsuccessful in eluting the enzyme from that portion of the gel by a variety of techniques.

The experiments reported here were carried out with the DEAE-purified enzyme unless specifically noted.

Polyacrylamide Gel Electrophoresis. The results of the electrophoresis of the mitochondrial KCl extract at pH 8.9 and 4.5 are shown diagrammatically in Figure 3a and c, respectively. At pH 8.9, the KCl extract was separated into five prominent and three lighter bands which agrees well with the number of separate peaks observed on the DEAE column run at pH 8.0. Figure 3b shows a single protein band

TABLE III: Relative Rates of Hydrolysis of Nucleotides by KCl Extract and Partially Purified Nucleoside Triphosphatase from Rat Liver Mitochondria.[a]

Compound	Relative Rate of Hydrolysis	
	KCl Extract	Partially Purified Enzyme
	%	%
ATP	100	100
CTP	80	69
GTP	79	79
UTP	78	78
dATP	103	124
dCTP	92	101
dGTP	44	101
TTP	93	67
ADP	49	26
dCDP		25
p-Nitrophenyl phosphate	79	72

[a] ATP hydrolysis was taken as 100%. The assay conditions and inorganic phosphate determination are described in Methods.

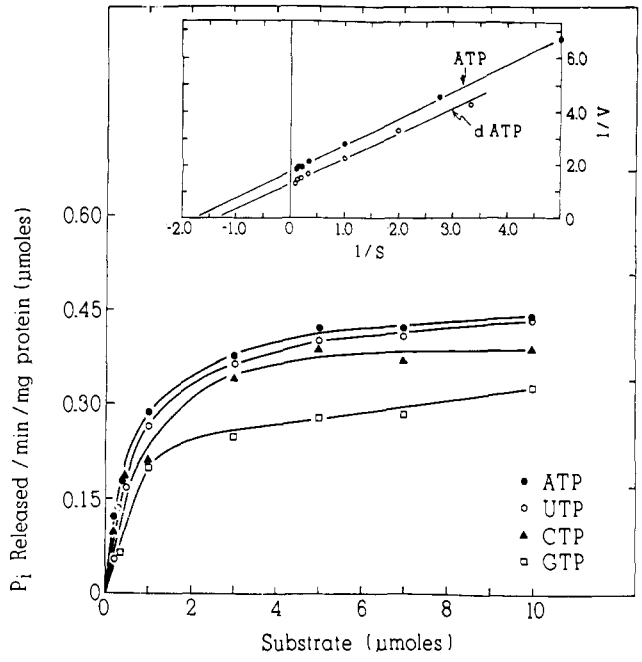

FIGURE 5: Effect of substrate concentration on the activity of the mitochondrial acid nucleoside triphosphatase, using ribonucleoside triphosphates. The enzyme assay and inorganic phosphate determination were carried out as described in Methods: (●——●) ATP; (▲——▲) CTP; (□——□) GTP; (○——○) UTP. The insert presents two representative Lineweaver–Burk plots: (●——●) PAT; (○——○) dATP.

to be present in the gel when the DEAE-purified nucleoside triphosphatase was electrophoresed under the same conditions. Because the enzyme became inactivated after 1 hr at this pH, even at 4°, no band at ATPase activity could be correlated with the protein bands. Electrophoresis of the KCl extract at pH 4.5 and 4° resulted in an electrophoretogram which contained five protein bands (Figure 3c). When the KCl extract was run under the same conditions and then assayed for ATPase activity, a single fast-moving band was observed (Figure 3d). Furthermore, the purified enzyme showed a single band of ATPase activity when run under identical conditions (Figure 3e).

Effects of Enzyme Concentration. The rate of nucleoside triphosphate hydrolysis was proportional to the enzyme concentration used for assay (20–180 μg).

Time Course of the Reaction. The hydrolysis of the various nucleoside triphosphates, under the standard conditions of incubation and assay, was observed to be linear for at least 60 min.

Effect of pH on Activity. As can be seen from Figure 4, the mitochondrial nucleoside triphosphatase had an optimal pH at 5–5.5 when ATP was the substrate. The same pH optimum was observed with all of the ribonucleoside and deoxyribonucleoside triphosphates. The enzyme showed virtually no activity in the range 7.5–9.0 in the presence or absence of either Mg^{2+} or Ca^{2+}.

Although not shown in Figure 4, the pH curve obtained when Fe^{2+} was present in the incubation mixture showed the same shape, but the pH optimum was shifted 0.5 unit to pH 6.

Substrate Specificity. The KCl extract of mitochondrial inner membrane–matrix preparations exhibits good phosphohydrolytic activity toward ribo- and deoxyribonucleoside triphosphates and in addition shows activity toward the diphosphates. As stated earlier, the extract is inactive toward nucleoside monophosphates and sodium pyrophosphate. The extract also shows activity on *p*-nitrophenyl phosphate. The DEAE-purified enzyme shows the same substrate specificity as the extract as can be seen in the data presented in Table III; the data are expressed as the rate of hydrolysis of a given substrate relative to the rate of hydrolysis of ATP which is assigned a value of 100%. Of the nucleoside triphosphates tested, dATP was the most active substrate. The diphosphates are represented by ADP and dCDP.

The products of the reaction are a nucleoside diphosphate and P_i. The enzyme does not appear to carry out a pyrophosphorolysis of the nucleoside triphosphate into a nucleoside monophosphate and pyrophosphate. Such a reaction would require the further splitting of the pyrophosphate into P_i either by the nucleoside triphosphatase itself or some contaminating pyrophosphatase before it would be picked up in the enzyme assay. That this is not the case is indicated by the fact that both the extract and the partially purified enzyme are inactive toward sodium pyrophosphate.

Effect of Substrate Concentration. Figure 5 shows the relationship of activity to substrate concentration for ribonucleoside triphosphates. Similar curves, not presented in Figure 5, were obtained with the deoxyribonucleoside triphosphates. The maximal rate of hydrolysis for all of the nucleoside triphosphates occurs at a substrate concentration of approximately 5 mM. The apparent K_m values (Table IV) were estimated from Lineweaver–Burk plots of the reaction velocities at various concentrations of nucleoside triphosphates. Two representative plots are presented in Figure 5

TABLE IV: Kinetics of Hydrolysis of Nucleoside Triphosphates by Purified Acid Nucleoside Triphosphatase from Rat Liver Mitochondria.[a]

Substrate	K_m (M × 10^{-4})	V_{max} (μmoles of P_i/mg per min)
ATP	6.2	0.47
CTP	6.5	0.43
GTP	4.4	0.31
UTP	7.8	0.55
dATP	8.3	0.67
dCTP	10.1	0.65
dGTP	7.4	0.49
TTP	5.7	0.38

[a] The enzyme assay conditions and procedure for determination of inorganic phosphate are described in Methods.

(insert). The activities with all the nucleoside triphosphates tested followed linear Lineweaver–Burk kinetics in the substrate concentration ranges used.

Effects of Activators and Inhibitors. INORGANIC IONS. Most ATPases and other nucleotidase preparations from mammalian sources are activated by metal ions, particularly Mg^{2+} or Mn^{2+}. The data in Table V illustrate the effect of inorganic ions on the hydrolysis of ATP by the rat liver mitochondrial nucleoside triphosphatase. It can be seen that the mitochondrial enzyme, unlike other ATPases and mammalian nucleotidases, is inhibited by Mg^{2+}, Ca^{2+}, and Mn^{2+}. Both Mg^{2+} and Ca^{2+} show no effect at levels below 1 mM but become increasingly inhibitory at higher concentrations. Other cations such as Ni^{2+}, Cu^{+}, and Cu^{2+} are also inhibitory at 10^{-3} M. Ammonium molybdate and sodium fluoride are good inhibitors of the enzyme. Although only the effect of these ions on ATP is presented in Table V, they inhibited the enzyme regardless of the substrate used.

TABLE V: Effect of Inorganic Ions on Activity of Mitochondrial Acid Nucleoside Triphosphatase.[a]

Compound	Concentration (M)	Inhibition (%)
None		0
Mg^{2+}	10^{-3}	0
	5×10^{-3}	29
	10^{-2}	46
	5×10^{-2}	68
Ca^{2+}	10^{-3}	0
	5×10^{-3}	20
Mn^{2+}	3×10^{-3}	69
Ni^{2+}	10^{-3}	19
Cu^{+}	10^{-3}	40
Cu^{2+}	10^{-3}	45
F^{-}	5×10^{-3}	81
Ammonium molybdate	5×10^{-3}	80

[a] Activity was determined in a 1-ml reaction mixture containing 5 mM Mg^{2+}-free ATP, 50 mM acetate buffer, pH 5.5, 0.4 mM 2-mercaptoethanol, 100 μg of enzyme protein, and inorganic ions at the concentrations indicated. Inorganic phosphate was determined as described in Methods.

TABLE VI: Effect of Iron on Nucleoside Triphosphatase Activity.[a]

Experiment		Triphosphatase Activity (μmole of P_i/mg of protein/min)
1	Complete system	0.20
	Complete system + Fe^{2+} (1 μmole)	0.39
	Complete system + Fe^{3+} (2 μmoles)	0.08
	Complete system + ascorbic acid (1 μmole)	0.73
	Complete system + ascorbic acid (5 μmoles)	0.73
	Complete system + Fe^{3+} (2 μmoles) + ascorbic acid (5 μmoles)	0.18
2	Complete system	0.21
	Complete system + ascorbic acid (0.1 μmole)	0.40
	Complete system + ascorbic acid (0.3 μmole)	0.80
	Complete system + ascorbic acid (0.5 μmole)	0.80
	Complete system + Fe^{3+} (1 μmole)	0.10
	Complete system + $FeCl_2$ (1 μmole)	0.33
	Complete system + $FeSO_4$ (1 μmole)	0.43
	Complete system + Fe^{3+} (1 μmole) + 2-mercaptoethanol (0.4 μmole)	0.23

[a] The enzyme assays and P_i determinations were carried out as described in Methods. Enzyme protein (100 μg) was used and other additions were made at the level described in the table.

It has been observed that 10^{-3} M Fe^{2+} is capable of stimulating the enzyme activity two- to fivefold depending upon the preparation and the enzyme fraction employed in the assay. Table VI presents data which illustrate the effect of iron on nucleoside triphosphatase activity. It can be seen that 10^{-3} M Fe^{2+} stimulates the activity twofold, whereas the same concentration of Fe^{3+} causes a 50% inhibition. The inhibition by Fe^{3+} can be overcome by reducing it to Fe^{2+} with low levels (3×10^{-4} M) of ascorbic acid. A reversal of inhibition by Fe^{3+} can also be effected by 2-mercaptoethanol (4×10^{-4} M) (Table VI, expt 2). Furthermore, the

addition of ascorbic acid to the enzyme system in the absence of exogenous Fe^{3+} also causes a considerable stimulation of activity (Table VI).

The amount of iron relative to nucleoside triphosphate required to bring about optimal stimulation has been determined for a number of substrates and has been found to be 1 μmole of Fe^{2+} per 5 μmoles of nucleoside triphosphate.

Organic Compounds. The enzyme was stimulated by sulfhydryl compounds such as 2-mercaptoethanol, glutathione, cysteine, and dithiothreitol (Table VII); mercaptoethanol had the greatest effect. As the enzyme was purified, the effect of mercaptoethanol became less pronounced. EDTA (5.6 mM) produced a variable but always slight inhibition. L-(+)-Tartrate had no effect on the activity.

Inhibitors of sulfhydryl enzymes such as iodoacetamide, iodoacetic acid, *N*-ethylmaleimide, and *p*-chloromercuribenzoate were found to be inhibitory to the nucleoside triphosphatase at 10^{-3} M, but only in the presence of mercaptoethanol. It has not yet been determined whether this inhibition is merely due to an interaction of the inhibitor with the added mercaptoethanol, thereby preventing it from stimulating enzyme, or with the enzyme *per se*.

Stability to Heat. In order to ascertain whether the phosphohydrolytic activity toward the ribo- and deoxyribonucleoside triphosphates observed in the KCl extract reflected the activity of a single enzyme or several enzymes, the sensitivity of the activity to increased temperature was studied. Aliquots of the KCl extract were heated for 30 min in water baths stabilized at 45, 60, and 75° and the enzyme was assayed for activity on ribonucleoside and deoxyribonucleoside triphosphates. There was virtually no activity on any of the substrates tested after the dilute enzyme (1 mg/ml) was heated for 30 min at 75°. Exposure at 60° for 30 min resulted in a 60–70% loss in activity toward all substrates. After 30 min at 45°, the loss of activity ranged between 10 and 30%. Between 45 and 75°, the rates at which the activity toward the various substrates decreased by the heat treatment were virtually identical.

TABLE VII: Effect of Organic Compounds on Activity of Mitochondrial Acid Nucleoside Triphosphatase.[a]

Compound	Concentration (M)	% Change
None		0
2-Mercaptoethanol	10^{-4}	+29
	5×10^{-4}	+105
	10^{-3}	+95
Glutathione	10^{-3}	+68
	5×10^{-3}	+108
Cysteine	10^{-3}	+22
	5×10^{-3}	+22
Dithiothreitol	4×10^{-4}	+54
EDTA	5.6×10^{-3}	−(5–18)
L-(+)-Tartrate	10^{-4}	0

[a] Activity was determined in a 1-ml reaction mixture containing 5 mM Mg^{2+}-free ATP, 50 mM acetate buffer, pH 5.5, 0.4 mM 2-mercaptoethanol, 100 μg of enzyme protein, and organic compounds at the concentrations indicated. Inorganic phosphate was determined as described in Methods.

Discussion

These studies provide evidence for the presence of a soluble nucleoside triphosphatase in rat liver mitochondria. A similar activity has been extracted from mitochondria of rabbit and lamb heart and rabbit kidney (G. F. Kalf and M. A. Gréce, unpublished experiments).

The enzyme appears to be of mitochondrial origin because the ratio of ATPase and dATPase activity at pH 5.5 to the activity of the mitochondrial marker enzyme, succinic dehydrogenase, approached a constant value after five washes (Table I). Although a KCl extract of *whole* mitochondria contained some slight activity toward substrates such as AMP, glucose 6-phosphates, and β-glycerophosphate and, therefore, probably contained contaminating cytoplasmic enzymes, extracts of inner membrane–matrix preparations and the DEAE column purified enzyme were active only on ribo- and deoxyribonucleoside di- and triphosphates and *p*-nitrophenyl phosphate.

The nucleoside triphosphatase from mitochondria appears to be different from the ATPases A and B isolated from nuclei (Fischer *et al.*, 1959; Siebert and Humphrey, 1965) on the basis of its optimal pH of 5.5 (Figure 2), its inhibition by concentrations of Mg^{2+} (Table V) which are optimal (3×10^{-3} M) for the activation of the nuclear ATPase A, and the lack of activity observed with the mitochondrial enzyme between pH 8 and 9 in the presence or absence of Ca^{2+} or Mg^{2+}.

The mitochondrial enzyme can also be distinguished from the crystalline acid phosphatase (Igarashi and Hollander, 1968) and the lysosomal acid nucleotidase both of which have been isolated from rat liver (Arsenis and Touster, 1968) by the difference in substrate specificity and the lack of inhibition of the mitochondrial nucleoside triphosphatase by L(+)-tartrate (Table VII).

Evidence was obtained which suggested that a single phosphatase was responsible for the broad substrate specificity. This conclusion is supported by the following facts. (a) The pH optimum is similar for each of the nucleoside triphosphates. (b) Analysis of fractions from the DEAE column for activity on each of the nucleoside triphosphates yielded single coincident peaks. (c) The hydrolysis of all substrates is inhibited to the same extent by fluoride. (d) The rates of thermal inactivation between 45 and 75° were similar for all of the ribo- and deoxyribonucleoside triphosphates. (e) Electrophoresis on polyacrylamide gels, under both acidic and basic conditions, indicated the presence of only one protein band in the chromatographically purified enzyme preparation (Figure 3). (f) The ATPase:dATPase ratio remained constant throughout the purification of the enzyme.

Most mammalian ATPases and nucleoside triphosphatases studied thus far appear to require Mg^{2+} or Mn^{2+}. The acid ATPase isolated from chloroplasts of *Euglena* (Carell and Kahn, 1967) also requires divalent cations but the enzyme from spinach chloroplasts does not (Young and Packer, 1966). The acid nucleoside triphosphatase studied in this report, however, did not require these metal ions and in fact

was inhibited by rather low levels (1 mM) of the usual divalent cation. Compatible with this is the fact that L-(+)-tartrate, an inhibitor of a number of acid phosphatases, did not affect the mitochondrial enzyme.

Fluoride on the other hand inhibited the reaction 80% at concentrations of 5 mM. It is possible that the fluoride ion inhibition of the nucleoside triphosphatase may be the result of a mechanism other than the formation of a magnesium–fluoro–phosphate complex in the presence of the released inorganic phosphate (Warburg and Christian, 1941), although this point has not been investigated as yet. For example, fluoride is known to inhibit 5′-adenylic acid deaminase (Nikiforuk and Colowick, 1955) by forming an inhibitory adenylate complex; the inhibition is independent of the presence of metals and the combination of the fluoroadenylate complex with the enzyme is dependent on the presence of certain positively charged groups on the protein and is therefore a pH-dependent characteristic.

Although the nucleoside triphosphatase showed considerable activity in the absence of exogenous cations, this activity was considerably stimulated by the addition of 10^{-3} M Fe^{2+}. The activity observed in the absence of added iron might be due to the presence of Fe^{2+} tightly bound to the enzyme. That this might be the case and that a portion of the bound iron might be in the inhibitory Fe^{3+} form is indicated by the ability of low levels of both 2-mercaptoethanol and ascorbic acid to stimulate the enzyme activity in the absence of added Fe^{2+}. Inhibition by EDTA was variable, but never more than 18%.

The inability of sulfhydryl inhibitors such as *N*-ethylmaleimide and *p*-chloromercuribenzoate to inhibit the nucleoside triphosphatase activity in the absence of mercaptoethanol, together with the requirement for nonheme iron, confer upon the nucleoside triphosphatase certain properties similar to those of the "ferredoxin-type" proteins (Malkin and Rabinowitz, 1967).

In reflecting on a possible biological function for the nucleoside triphosphatase in rat liver mitochondria, it is of interest that ATPase with similar properties has been isolated from chloroplasts of *Euglena gracilis* (Carell and Kahn, 1967). In rapidly dividing heterotrophically grown cells, photosynthetic activity and chlorophyll formation are suppressed, whereas the Mg^{2+}-dependent ATPase (dATPase) is at a maximum. However, as the culture approaches maximal growth, the Mg^{2+}-dependent ATPase activity declines to a minimum with a concomitant rapid increase in the photosynthetic activity. Carell and Kahn postulate that the enzyme has a function in the control of the formation of the photosynthetic apparatus by regulating, through inhibition, the replication of nucleic acids required for chloroplast development. It is interesting to speculate that the mitochondrial nucleoside triphosphatase might function in an analogous manner to control the replication of mitochondrial nucleic acids and thereby regulate the biosynthesis of the mitochondrial membrane.

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