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Purification and Properties of Nucleoside Triphosphate–Adenosine Monophosphate Transphosphorylase from Beef Heart Mitochondria*

G. J. Albrecht

ABSTRACT: A simple method for preparing mitochondrial nucleoside triphosphate–adenosine monophosphate transphosphorylase, purified about 40-fold, is described. The enzyme seems to be fairly stable under different conditions, but optimum activity occurs at pH 8.5. Ethylenediaminetetraacetic acid, glutathione, cysteamine, and high inorganic phosphate concentrations have no effect on its activity. The molecular weight of the enzyme is estimated to be about 52,000, and an ultraviolet spectrum is given. The reversible incorporation of [γ-^{32}P]guanosine triphosphate and [^{14}C]guanosine triphosphate shows that the enzyme forms a nucleotide–enzyme complex and not a phosphorylated enzyme intermediate. K_M and K_i values for different substrates show that the enzyme is nonspecific toward nucleoside triphosphates. However, there is a specificity for adenosine monophosphate. The substrate specificity, expressed in V_{max}, is determined by assay and by calculation from kinetic studies. In the direction of adenosine monophosphate phosphorylation all substrates tested inhibit competitively. Adenosine diphosphate and guanosine diphosphate are noncompetitive inhibitors in the reverse reaction. The enzyme is stimulated by metal ions, optimally by 4.0×10^{-3} magnesium. The nucleoside triphosphate–adenosine monophosphate transphosphorylase seems to follow a sequential type of reaction mechanism in both directions. Two active sites of the enzyme (for nucleotide alone and for nucleotide magnesium complex) are proposed.

A large number of nucleotides in their different phosphorylated stages were shown to occur in mitochondria (Siekevitz and Potter, 1955; Heldt, 1966). Only the adenosine nucleotides seem to be immediate products of the oxidative phosphorylation. Most of the other nucleotides are derived from these by coupled phosphorylation at the substrate level (Slater and Holton, 1953; Sanadi *et al.*, 1954, 1955; Heldt *et al.*, 1964; Rossi and Gibson, 1964; Lardy *et al.*, 1965). One of the most important steps in nucleotide phosphorylation is carried out by the nucleoside phosphate kinases (Strominger *et al.*, 1954; Lieberman *et al.*, 1954; Herbert *et al.*, 1955; Gibson *et al.*, 1956).

In mitochondria, over 90% of the endogenous AMP is phosphorylated by these enzymes (Slater and Holton, 1953). The adenosine-specific adenylate kinase (EC 2.7.4.3) can be substituted by the nonspecific nucleoside triphosphate–adenosine monophosphate transphosphorylase (EC 2.6.4.d) which uses other nucleoside triphosphates as substrates for the phosphorylation of AMP. It was described and partially purified by Gibson *et al.* (1956), Strominger *et al.* (1954, 1959), Heppel *et al.* (1959), and Chiga *et al.* (1961), from cell homogenates. Heldt and Schwalbach (1967) showed that the enzyme is located in mitochondria.

In this report the purification, some properties, and the kinetics of the mitochondrial NTP–AMP transphosphorylase are described. The enzyme is compared with the mitochondrial adenylate kinase and nucleoside triphosphate–nucleoside diphosphate kinase (EC 2.7.4.6). All three enzymes seem to be most important in the regulation of the mitochondrial adenosine nucleotide level.

Experimental Procedure

Reagents. Nucleotides, substrates, and most enzymes used were obtained from C. F. Boehringer & Soehne, GmbH, Mannheim, Germany. The hexokinase, type III (EC 2.7.1.1), was a preparation from Sigma Chemical Co., St. Louis, Mo.; the NTP–NDP kinase used occurred in a sufficient amount as an impurity in this same preparation. [γ-^{32}P]GTP was prepared by Dr. H.-W. Heldt, Department of Physical Biochemistry, University of Munich, and was generously donated for these experiments.

Protein Determination. The protein content in the suspension of mitochondria and in the enzyme extracts was deter-

* From the Institut für Angewandte Botanik der Technischen Hochschule München, Munich 2, Germany. *Received June 13, 1969.* Send reprint requests to this address. This work was supported by a fellowship and a grant from the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany. All experiments were done in the Institute for Physiological Chemistry, University of Munich, Munich, Germany. The abbreviations used are those recommended by the International Congress of Biochemistry, 1964, and IUPAC, 1957.

mined by a modified Biuret method (Kröger and Klingenberg, 1966). In small volumes of enzyme solution the protein was measured by the procedure of Warburg and Christian (1942).

Measurement of Transphosphorylase Activities. The activities of adenylate kinase, NTP–NDP kinase, and NTP–AMP transphosphorylase were measured with the following assays.

$$ATP + AMP \rightleftharpoons ADP + ADP$$

$$NTP + ADP \rightleftharpoons NDP + ATP$$

$$NTP + AMP \rightleftharpoons NDP + ADP$$

Assay A (Heldt and Schwalbach, 1967). The assay mixture contained in a final volume of 0.5 ml: 25 μmoles of triethanolamine-HCl buffer (pH 8.0), 0.5 μmole of EDTA, 2 μmoles of Mg^{2+}, 10 μmoles of glucose, 0.1 μmole of NADP, 0.25 μmole of ADP, 0.25 unit of hexokinase (type III), 0.12 unit of glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and enzyme extract. For the assay of the NTP–AMP transphosphorylase, 0.25 μmole of GDP was added.

To measure NTP–NDP kinase in the extract with this assay, 0.25 μmole of GTP instead of GDP and hexokinase (Boehringer) instead of hexokinase (type III from Sigma) were used. In order to repress the adenylate kinase activity, 5 μmoles of AMP was added.

Assay B (Strominger *et al.*, 1954). This assay was primarily applied to test the activities of adenylate kinase and of NTP–AMP transphosphorylase in more purified preparations. The test mixture contained in a final volume of 0.5 ml: 25 μmoles of triethanolamine-HCl buffer (pH 8.5), 2 μmoles of Mg^{2+}, 37.5 μmoles of KCl, 0.5 μmole of EDTA, 1.25 μmoles of phosphoenolpyruvate, 3.75 μmoles of NADH, 0.12 μmole of AMP, 0.25 μmole of GTP, 0.75 unit of pyruvate kinase (EC 2.7.1.40), 0.45 unit of lactate dehydrogenase (EC 1.1.1.28), and enzyme extract.

The pyruvate kinase reacts not only with ADP but also with GDP, IDP, UDP, and CDP, although at different rates. In all cases, 1 μmole of NTP used was considered equivalent to 2 μmoles of NADH oxidized. Assays A and B yielded comparable enzyme activities.

Assay C (Adam, 1961). This assay was used to measure AMP formation. The test mixture contained in a final volume of 0.5 ml: 25 μmoles of triethanolamine-HCl buffer (pH 8.5), 2 μmoles of Mg^{2+}, 0.25 μmole of hydrazine sulfate, 0.25 μmole of 3-phosphoglycerate, 3.75 μmoles of NADH, 0.25 μmole of ADP, 0.25 μmole of NDP, 0.45 unit of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (a crystalline suspension of the enzyme was centrifuged, and the crystals were dissolved in an equal volume of 0.05 triethanolamine-HCl buffer (pH 8.5) containing 3 mg of GSH/ml), 2.25 units of 3-phosphoglycerate kinase (EC 2.7.2.3), and enzyme extract.

A control was first run and then the reactions were started with 5–10 μl of enzyme extract, equivalent to 0.5–1.0 unit (assay B) or 0.1–2.0 units (assay A and C)). The change in optical density according to the formation of NADPH from $NADP^+$ or NAD^+ from NADH was recorded at 366 or 334 nm, respectively, in a 1-cm cuvet.

Purification of the NTP–AMP Transphosphorylase

Preparation of Beef Heart Mitochondria. The isolation of mitochondria from beef heart followed a modified procedure by Klingenberg and Slenzka (1959). The isolation medium was 0.25 M sucrose–0.01 M Tris–0.002 M EDTA (pH 7.5).

Fresh beef hearts were obtained from the slaughter house, cooled immediately in the ice-cold isolation medium, freed from fat, skin, and tendons in a cold room, and cut into small pieces and ground. The ground meat was homogenized in the isolation medium with a Braun Multimix. The resulting suspension was readjusted to pH 7.5 with 3.0 N KOH and remixed for 20 sec. Cell debris was removed by centrifugation at 500*g* for 15 min at 0°. Subsequent centrifugation at 10,000*g* and 0° for 20 min yielded the mitochondrial fraction. The pooled mitochondria were washed twice by resuspending them with a Potter-Elvehjem homogenizer in fresh isolation medium and by centrifugation as before. At this point the mitochondria could be stored in ice overnight.

Extraction and Isolation of the NTP–AMP Transphosphorylase. Extraction of the mitochondria with PO_4 buffer. All steps were carried out at 4°, unless otherwise stated. The mitochondria were suspended in 0.075 M sodium phosphate buffer (pH 7.5), and left in ice for several hours (preferably overnight); 100% of the NTP–NDP kinase and more than 90% of adenylate kinase were extracted from the mitochondria by washing them five times. The NTP–AMP transphosphorylase could not be extracted.

Extraction of the enzyme by sonification. The adenylate kinase- and NTP–NDP kinase-depleted mitochondria were resuspended (about 20 mg of protein/ml) in 0.075 M PO_4 buffer (pH 7.5). They were disrupted in small centrifuge tubes by sonification (three times for 10 sec) with a Branson sonifier (Model S-75) at maximum output. The particles were removed by centrifugation at 125,000*g* for 45 min. The supernatant (fraction I) contained all NTP–AMP transphosphorylase activity.

First ammonium sulfate precipitation. Fraction I was brought to 30% ammonium sulfate saturation (Noda and Kuby, 1957) with solid salt. The pH was maintained at 7.5 with 1 N KOH throughout this procedure. After stirring for 30 min, the supernatant from a 15,000*g* centrifugation (15 min) was then brought to 75% ammonium sulfate saturation. The centrifuged precipitate was taken up in a small amount of 0.005 M triethanolamine-HCl buffer (pH 7.5) and dialyzed against two changes of 5–10 l. of the same buffer overnight. A heavy precipitate was removed. The supernatant (fraction II) contained the NTP–AMP transphosphorylase without greater loss of activity.

DEAE-cellulose. Prior to chromatography, the DEAE-cellulose was washed with 1 N NaOH and with 0.005 M triethanolamine-HCl buffer (pH 7.5). A column (from 1 × 15 to 2.5 × 50 cm) was poured and washed with 0.005 M triethanolamine-HCl buffer (pH 7.5) until the eluate showed the same pH. Then fraction II was washed through the column with the same buffer. The NTP–AMP transphosphorylase activity was not absorbed and came off the column in a broad protein peak (fraction III).

Second ammonium sulfate precipitation. Fraction III protein was precipitated by addition of solid ammonium sulfate (65% saturation) and collected by centrifugation. The pellet was taken up in the smallest possible volume of 0.005 M

TABLE I: Purification Procedure of NTP–AMP Transphosphorylase from Beef Heart Mitochondria.[a]

Step	Vol (ml)	Total Protein (mg)	Adenylate Kinase		NTP–AMP Transphosphorylase				
			Total Units (μmoles/min)	Sp Act. (units/mg of protein)	Total Units (μmoles/min)	Sp Act. (units/mg of protein of extract)	Sp Act. (units/mg of protein of mitochondria)	-fold	Yield
1. Mitochondria	3,900	51,400							
2. PO_4 extracts	11,000	6,100	25,300	4.50	350	0.058	0.0068		
3. Mitochondria	3,200	43,200							
4. Sonic extract	2,200	6,800	2,900	0.43	22,500	3.30	0.44	1	100
5. 75% $(NH_4)_2SO_4$, dialyzed	350	4,100	2,000	0.49	21,500	5.55	0.42	1.68	95
6. DEAE-cellulose	230	560	2,000	3.71	17,700	31.6	0.34	9.60	79
7. 65% $(NH_4)_2SO_4$, dialyzed	5.7	200	1,500	7.50	12,700	64.0	0.25	19.40	57
8. Heat step, 3 min at 65°	5.0	97	1,000	11.30	12,600	135.5	0.24	41.00	56

[a] The data for adenylate kinase in the PO_4 extracts refer to the mitochondrial adenylate kinase–enzyme; the other numbers refer to the nonspecific adenylate kinase activity of the NTP–AMP transphosphorylase. For further details, see text.

triethanolamine-HCl buffer (pH 8.5) and dialyzed against the same buffer, containing 35% of ammonium sulfate (fraction IV).

HEAT STEP. Fraction IV was heated in a water bath to 65°, kept at this temperature for 3 min, and then rapidly cooled to 0° in ice. A heavy precipitate was centrifuged. The supernatant was fraction V.

Fraction V could be stored at 0° for several weeks without appreciable loss of NTP–AMP transphosphorylase activity. In at least three different preparations it showed the same specific activity; 55–60% of the originally extracted activity, purified about 40-fold, was obtained. Treatment with calcium phosphate gel, aluminum hydroxide gel, Sephadex or DEAE-Sephadex column chromatography, or IRC-50 ion-exchange resin resulted in no further purification. Attempts to crystallize the enzyme were unsuccessful. Polyacrylamide gel electrophoresis showed two very faint bands and a broad band from which a purity of $\geq 75\%$ of active protein could be estimated.

The purification of the NTP–AMP transphosphorylase is summarized in Table I. Fraction V, dialyzed against 0.05 M triethanolamine-HCl buffer (pH 8.5), was used for the experiments and kinetic studies described below.

Conclusions from the Purification Procedure. Over 90% of the adenylate kinase and all NTP–NDP kinase could be extracted with hypotonic PO_4 buffer. The latter enzyme was mainly in the third extract and showed a total activity of 3000 units and a specific activity of 0.48 (not shown in Table I).

The hypotonic PO_4 buffer causes a swelling of the mitochondria which seems to increase the permeability for adenylate kinase and NTP–NDP kinase. These observations led to the conclusion that these enzymes are located on the outer membrane and thus are easily extractable (Heldt, 1966; Markland and Wadkins, 1966; Klingenberg and Pfaff, 1966). On the contrary, the NTP–AMP transphosphorylase seems to be bound to the membrane in the intracristae space (Heldt and Schwalbach, 1967; Glaze and Wadkins, 1967; Lima *et al.*, 1968).

A longer period of sonification yielded no higher NTP–AMP transphosphorylase, but decreased the adenylate kinase. The NTP–AMP transphosphorylase occurs in a fairly high amount in beef heart mitochondria. Assuming that the purified enzyme has a final specific activity of about 135 units/mg of protein, it constituted approximately 2–3% of the mitochondrial protein extracted by sonification.

Protection of the enzyme during the heat step was important; 35% ammonium sulfate, AMP, or other suitable substrates were sufficient. After this step no increased instability of the enzyme could be found as stated in the characterization of the liver enzyme by Heppel *et al.* (1959).

Properties of the NTP–AMP Transphosphorylase. STABILITY. The stability of the enzyme increased with higher purity. Furthermore, the presence of ions (such as ammonium sulfate) or substrates seems to enhance the stability, even when the active protein was kept as a suspended precipitate. High dilutions, low ionic strengths, freezing and thawing of enzyme extracts, or mitochondria suspensions, decreased the activity rapidly. There was almost no loss of activity after a 1-week storage period at room temperature.

pH OPTIMUM. The NTP–AMP transphosphorylase is active over a broad range of pH, showing its highest activity at pH 8.5 (Figure 1). The connecting enzymes did not limit the assay at the pH values used.

ESTIMATION OF THE MOLECULAR WEIGHT. Three crystalline enzymes with known molecular weights were chromatographed on Sephadex G-200 together with the highly purified NTP–AMP transphosphorylase: alcohol dehydrogenase (EC 1.1.1.2), hexokinase, and cytochrome *c*. According to the method of Andrews (1964), the logarithms of the molecular weights were plotted against the elution volumes of the pro-

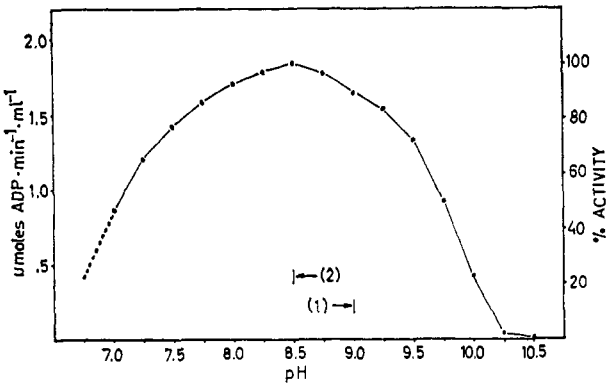

FIGURE 1: pH optimum curve of the NTP–AMP transphosphorylase. The data were obtained with assay B as described under Experimental Procedure. After the complete test mixture was adjusted to the desired pH, the reaction was started with enzyme. Two buffer systems were used: (1) 0.05 M Tris-HCl buffer (pH 7.0–9.0) and (2) 0.05 M glycine–KOH buffer (pH 8.5–10.5).

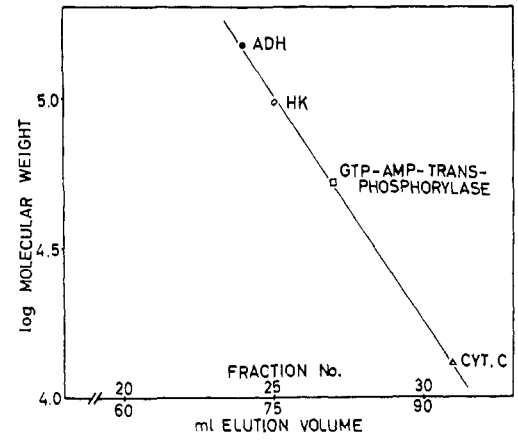

FIGURE 2: Estimation of the molecular weight of the NTP–AMP transphosphorylase by Sephadex gel G-200 filtration. Crystalline alcohol dehydrogenase (4.5 mg), crystalline hexokinase (5.0 mg), crystalline cytochrome c (5.0 mg), and highly purified NTP–AMP transphosphorylase (4.0 mg) in 0.2 M potassium phosphate buffer (pH 7.5; total volume 1.5 ml) were layered onto a Sephadex G-200 column (1.75 × 45 cm) previously equilibrated with 0.2 M $K\text{-}PO_4$ buffer (pH 7.5). The proteins were eluted with the same buffer. Fractions (1.5 ml) were collected, and the protein peaks coming off the column were assayed for activity in each individual fraction. For the determination of the molecular weight of the NTP–AMP transphosphorylase the following molecular weights were applied: alcohol dehydrogenase, 150,000; hexokinase, 96,600; and cytochrome *c*, 13,000.

tein peaks associated with the corresponding enzyme activities. A molecular weight calibration curve could be obtained as shown in Figure 2. The molecular weight of the NTP–AMP transphosphorylase was estimated to be about 52,000. This number was used in later calculations.

ULTRAVIOLET SPECTRUM OF THE ENZYME PROTEIN. Figure 3 shows an ultraviolet spectrum of the purified enzyme. A_{280} was 0.175 and the $A_{280}:A_{260}$ ratio was 1.84. Characteristic maxima, *e.g.*, at 252.5 and 259 nm, correspond to the absorption maxima of guanosine and adenosine nucleotides, respectively. Other maxima designated at 3, 4, and 5 in Figure 3 might correspond, respectively, to those of uridine, cytosine, and inosine nucleotides which may be bound to the enzyme protein at different sites. For a myokinase spectrum, Noda and Kuby (1957) and Callaghan and Weber (1955) reported a maximum around 250 nm and shifting maxima between 279 and 265 nm. This range depends upon the concentrations of bound adenosine nucleotides. These findings could also apply to the NTP–AMP transphosphorylase, in accordance with myokinase where traces of nucleotides remain bound tenaciously to the enzyme throughout the process of purification (Callaghan and Weber, 1955).

INCORPORATION OF [γ-^{32}P]GTP AND [^{14}C]GTP INTO THE ENZYME PROTEIN. Considering the conclusions drawn from the ultraviolet spectrum, from kinetic studies, and from the reaction mechanism of the enzyme suggested below, it was presumed that the enzyme either forms a nucleotide–enzyme complex or is phosphorylated by its substrate to a phosphoenzyme intermediate. With this, the whole reaction would be brought to completion as reported with the mitochondrial NTP–NDP kinase (Pedersen, 1968).

In order to verify either of these assumptions, the purified NTP–AMP transphosphorylase was incubated in the presence of [γ-^{32}P]GTP and [^{14}C]GTP, and filtered through a Bio-Gel P-4 column. The protein could easily be separated from excess [γ-^{32}P]GTP and [^{14}C]GTP and magnesium (Figure 4).

With this experiment it was clear that the enzyme forms no phosphoenzyme intermediate, but a nucleotide–enzyme complex. In an experiment, carried out with [γ-^{32}P]GTP alone, 4.75% of the total radioactivity was associated with the enzyme activity and protein. This means that 1 mole of enzyme protein binds 0.4 mole of [^{32}P]GTP. In other experiments this value could not be exceeded.

REMOVAL OF THE [γ-^{32}P]GTP FROM THE LABELED ENZYME FRACTION. Table II shows that magnesium alone could partially deplete the enzyme, and that magnesium plus AMP removed all [γ-^{32}P]GTP from the protein.

The removed radioactivity was still nucleotide bound. This was demonstrated by its adsorption onto charcoal (Table III). Small amounts of [^{32}P]P_i detectable may have resulted from degradation of these nucleotides.

These findings suggest again that a nucleotide–enzyme complex exists.

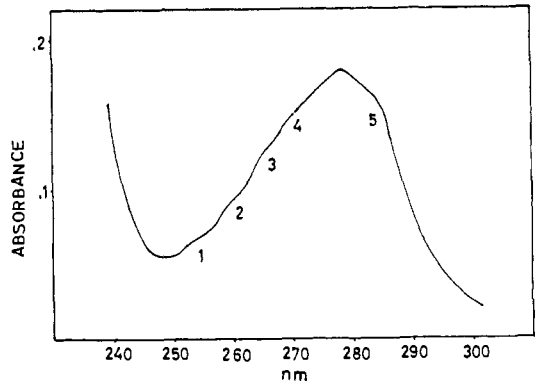

FIGURE 3: Ultraviolet spectrum of the NTP–AMP transphosphorylase. Enzyme protein (0.2 mg) in 1.0 ml of 0.05 M triethanolamine-HCl buffer (pH 8.5) was used to run a spectrum between 240 and 300 nm on a Hitachi spectrophotometer.

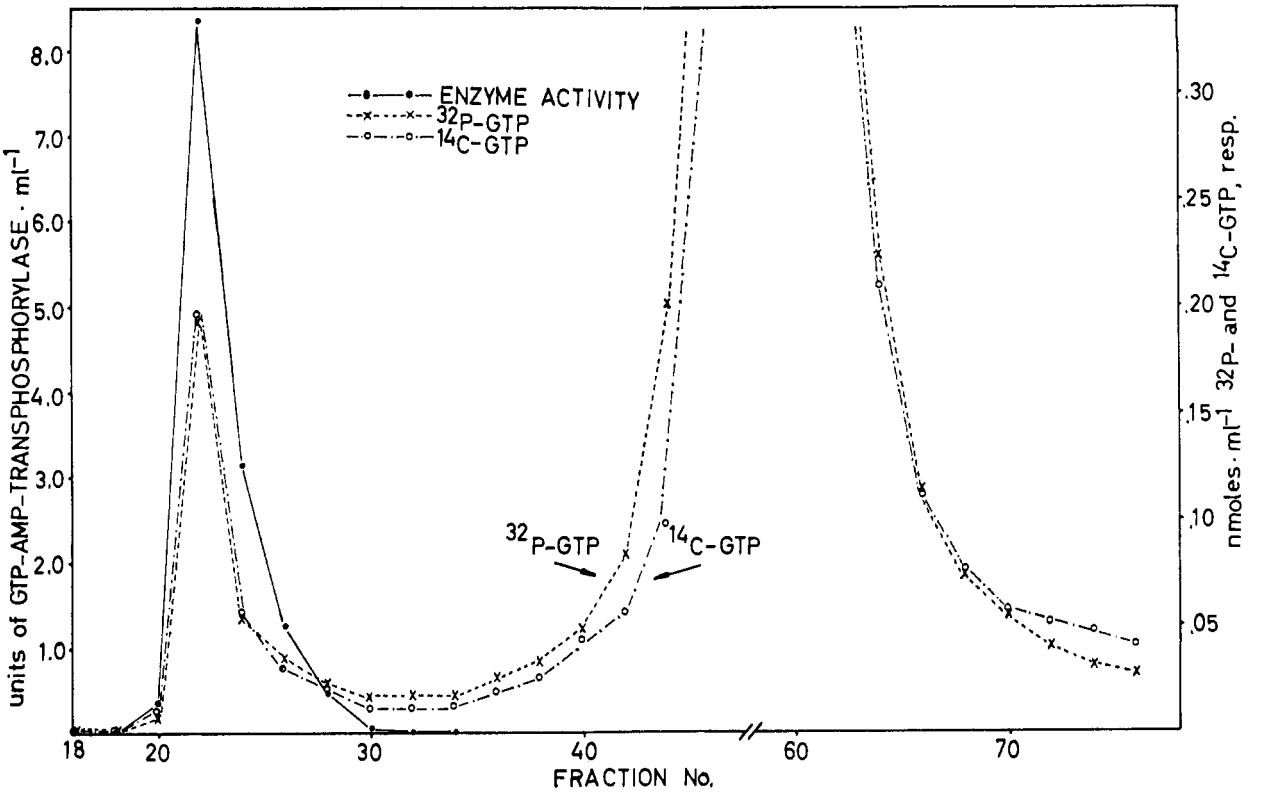

FIGURE 4: Incorporation of [γ-^{32}P]GTP and [^{14}C]GTP into NTP–AMP transphosphorylase. The incubation mixture contained in a final volume of 0.5 ml: 25 μmoles of triethanolamine-HCl buffer (pH 8.0), 1 mμmole of [γ-^{32}P]GTP, 14 mμmoles of [^{14}C]GTP; 50 mμmoles of carrier GTP, 2 μmoles of magnesium, and 0.238 mg of enzyme protein. The specific activity of [γ-^{32}P]GTP was 10,280 cpm/mμmole, that of [^{14}C]GTP was 8650 cpm/mμmole. After 10-min incubation at 30°, the sample was cooled to 0°, and after addition of 10 μmoles of EDTA layered immediately onto a column of Bio-Gel P-4 (100–200 mesh; 1.25 × 48 cm) previously equilibrated with 0.05 M triethanolamine-HCl buffer (pH 8.0). Fractions of 2 ml were collected and both the NTP–AMP transphosphorylase activity and the [γ-^{32}P]GTP and [^{14}C]GTP radioactivity were measured with assay B and with a Packard scintillation counter, respectively.

Kinetic Studies on the NTP–AMP Transphosphorylase

Phosphate Acceptors. With 3′-GMP, IMP, CMP, and UMP, the enzyme showed no activity at all. The only phosphate acceptor was AMP, indicating that the enzyme is specific for adenosine monophosphate, and for ADP in the reverse reaction.

Metal Ion Stimulation of the Enzyme. Divalent metal ions stimulated the NTP–AMP transphosphorylase. Magnesium showed its optimum stimulation at a concentration of 4.0×10^{-3} M; higher concentrations slightly inhibited the enzyme (Figure 5). Calcium produced about 50% of enzymatic activity (optimally at 4.0×10^{-3} M). Manganese yielded about the same activity as magnesium. The curve for manganese in Figure 5 could not be continued since at the pH of 8.5, con-

TABLE II: Removal of [γ-^{32}P]GTP from the Enzyme Protein.[a]

Additions	Moles of [γ-^{32}P]-GTP/Mole of Enzyme
None	0.42
+Mg^{2+} (4 mM)	0.09
+Mg^{2+} (4mM) +AMP (0.5 mM)	0.003

[a] [γ-^{32}P]GTP-labeled enzyme was incubated at 30° for 10 min with the indicated additions. Enzyme-bound [γ-^{32}P]-GTP was determined as described in Figure 4.

TABLE III: Identification of the Radioactivity Removed from the Enzyme Protein.[a]

Sample	cpm Applied	cpm Eluted	% Eluted
[^{32}P]P_i	182	164	90.4
From incubation without AMP	745	116	15.6
From incubation with AMP	824	162	19.6

[a] The second peak fractions of each indicated sample were individually pooled, and 1.0-ml portions were applied to small charcoal columns (0.3 × 1.0 cm) previously washed with water. The columns were eluted with 5.0 ml of water and the total radioactivity in the eluate was counted. A blank of [^{32}P]P_i was run in the same way for comparison of the yield in the eluate.

TABLE IV: Equilibrium Constant of the NTP–AMP Transphosphorylase.[a]

Expt	GTP		AMP		ADP ⇄ GDP		(GDP)(ADP)/ (GTP)(AMP) = K_{eq}
	Initial	Final	Initial	Final	Initial	Final	
1	2.42	0.91	4.39	2.88	0	1.51	0.870
2	4.52	1.89	7.19	4.54	0	2.65	0.818
3	4.52	2.42	4.47	2.35	0	2.12	0.792
4	2.42	0.68	7.26	5.53	0	1.74	0.806

[a] The test mixture contained in a final volume of 0.5 ml: 25 μmoles of triethanolamine-HCl buffer (pH 8.5), 2.5 μmoles of magnesium, 0.5 μmole of EDTA, sufficient enzyme (0.95 μg), and the nucleotides as given above in micromoles. After incubation at 25° for 20 min, the reaction was stopped by heating the sample to 100° for 5 min. GTP was measured with assay C, ADP with assay B, and AMP with assay B after addition of ATP and myokinase. All were measured at 366 nm.

centrations higher than 1.0 mM Mn^{2+} resulted in precipitation of both protein and white $Mn(OH)_2$.

Zinc, nickel, and trivalent iron even in a concentration of 4.0×10^{-3} M at this pH produced only poor activity.

Equilibrium Constant. The equilibrium constant of the enzyme reaction in the direction of AMP phosphorylation was determined to be 0.82 ± 0.029 as shown in Table IV.

Effect of Substrate Concentration on Homologous NTP–AMP Transphosphorylase Reactions. A number of homologous enzyme reactions, both forward and reverse (Figures 6 and 7), were run with assay B and C, respectively. One substrate concentration was kept constant while that of the other was progressively increased. In some cases an excess of 2.0 mM Mg^{2+} was used to test the possibility that a Mg–nucleotide complex was the true substrate. Increasing concentrations of AMP over a certain level led to an inhibition of the enzyme reaction, depending on the fixed concentration of GTP (Figure 6). However, an excess of Mg^{2+} reduced this level of AMP concentration and lowered the reaction rates (see also Figure 5).

When GTP was changed and AMP was kept constant at different levels, similar results occurred (not shown in Figure 6). The inhibiting concentration of GTP, however, was ten times higher than that of AMP. Excess of magnesium had only small effect; the reaction rates were lower than under optimum conditions.

The initial velocities from Figures 6 and 7 plotted according to the method of Lineweaver and Burk (1934) resulted in straight lines as shown in Figures 8 and 9. Similar curves were obtained when either GTP or GDP were the changeable substrates. Fans of straight lines were obtained, *i.e.*, the V_{max} and the K_M rise as the fixed substrate increases, suggesting a sequential type of reaction mechanism as proposed by Cleland (1963).

Substrate Affinities. The preceding studies made possible the selection of concentrations suitable for the determination of the Michaelis and inhibition constants. Curves plotted $1/v$ *vs.* $1/S$, and $1/v$ *vs.* I, respectively (Lineweaver and Burk, 1934), resulted in the data compiled in Table V and determined the kind of inhibition.

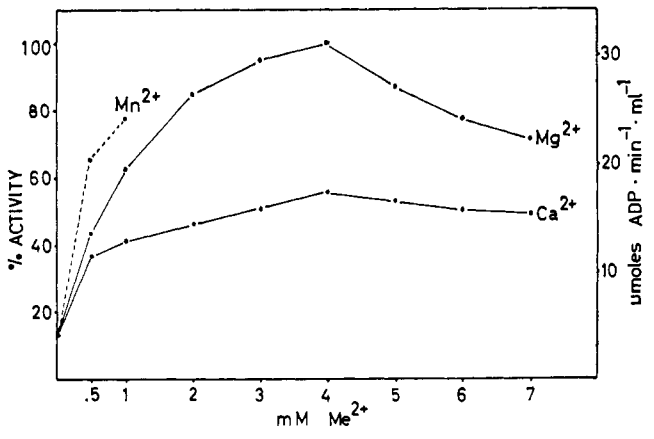

FIGURE 5: Metal ion stimulation of the NTP–AMP transphosphorylase. The test mixture contained in a final volume of 0.5 ml: 25 μmoles of triethanolamine-HCl buffer (pH 8.5), 0.25 μmole of GTP, 0.25 μmole of AMP, 1.0 μg of enzyme protein, and the indicated amounts of metal chlorides. Incubation time was 3 min at 25°. The reaction was stopped by heating the sample to 100° for 3 min. The formed ADP was measured with assay B at 334 nm.

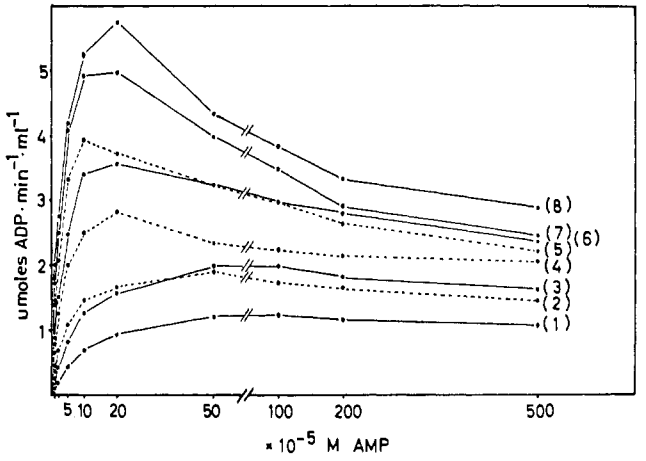

FIGURE 6: Effect of AMP concentration on homologous NTP–AMP transphosphorylase reactions. Assay B was used at pH 8.5, 25°, and 334 nm with a constant amount of enzyme. While the AMP concentration was changed, the GTP concentration was kept constant at: (1) 0.005 mM, (2) 0.02 mM + 2 mM Mg^{2+}, (3) 0.02 mM, (4) 0.05 mM + 2 mM Mg^{2+}, (5) 0.5 mM + 2 mM Mg^{2+}, (6) 0.05 mM, (7) 0.5 mM, and (8) 5.0 mM.

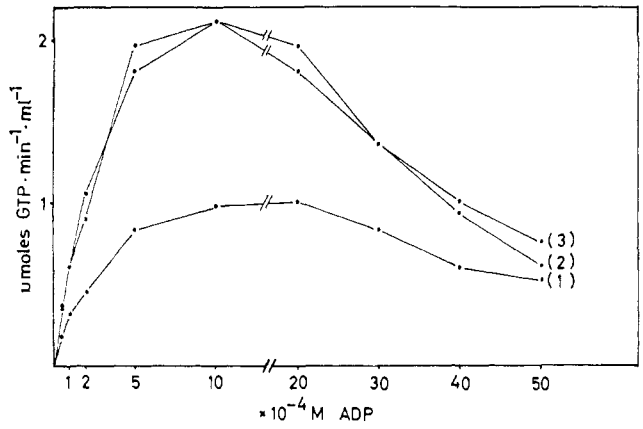

FIGURE 7: Effect of ADP concentration on homologous NTP–AMP transphosphorylase reactions. Assay C was used at pH 8.5, 25°, and 366 nm with a constant amount of enzyme. While the ADP concentration was changed, the GDP concentration was kept constant at: (1) 0.002, (2) 0.005, and (3) 0.01 mM.

Inhibition of the NTP–AMP Transphosphorylase. AMP and the nucleoside triphosphates tested were shown to be competitive inhibitors at high concentrations (probably mere substrate inhibitors, except ATP). GDP and ADP are definitively noncompetitive inhibitors. The K_M for GDP is very low compared with that for ADP. The K_M and K_I for ADP lie in the same exponential range. Conclusively, since these substrates seem to limit the enzyme reaction, the NTP–AMP transphosphorylase might constitute one of the regulatory enzymes in mitochondria.

Substrate Specificity. The maximum velocities of the enzyme with its substrates were obtained (1) from the points of intersection of the linear part of the curves with the ordinate,

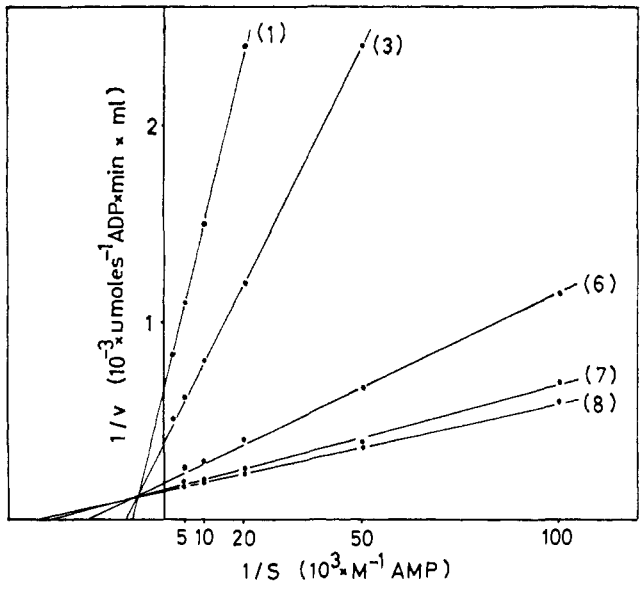

FIGURE 8: Effect of AMP concentration of homologous NTP–AMP transphosphorylase reactions. The initial velocities from Figure 6 were plotted according to the method of Lineweaver and Burk (1934). The numbers in the graph correspond to those concentrations given for Figure 6.

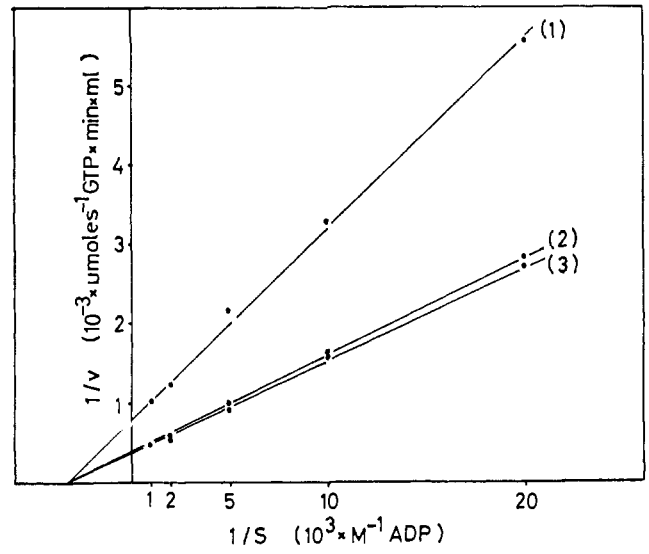

FIGURE 9: Effect of ADP concentration on homologous NTP–AMP transphosphorylase reactions. The initialv elocities from Figure 7 were plotted according to the method of Lineweaver and Burk (1934). The numbers in the graph correspond to those concentrations given for Figure 7.

and (2) from the relative reaction rates determined with assay B (Table VI). Both methods produced comparable data. The figures for V_{max}, however, are more reliable. The V_{max} for ATP differs significantly from that given for the highly specific adenylate kinase (Noda, 1958; Markland and Wadkins, 1966). The NTP–AMP transphosphorylase is clearly

TABLE V: Substrate Affinities and Inhibition of the NTP–AMP Transphosphorylase.[a]

Substrate	K_M	K_I	Type of Competition with Substrate
AMP	3.3×10^{-5}	1.6×10^{-3}	Competitive with GTP
GTP	5.6×10^{-5}	8.0×10^{-4}	Competitive with AMP
ITP	6.3×10^{-4}		
ATP	1.0×10^{-3}	7.4×10^{-4}	Competitive with GTP
		7.3×10^{-4}	Competitive with AMP
UTP	7.4×10^{-3}		
CTP	9.1×10^{-3}		
GDP	1.2×10^{-6}	7.7×10^{-4}	Noncompetitive with ADP
ADP	2.9×10^{-4}	9.0×10^{-4}	Noncompetitive with GDP

[a] The different substrates were tested with assay B and C, respectively. The K_M and K_I values were obtained by plotting the experimental data by the Lineweaver and Burk (1934) method and by extrapolating the linear part of the curves. The numbers are given in moles per liter.

TABLE VI: Substrate Specificity of the NTP–AMP Transphosphorylase.[a]

	Assay B		V_{max} from Lineweaver–Burk Plots		
Substrate	μmoles/min	%	μmoles/min	moles Utilized of NTP × min^{-1} × $mole^{-1}$ of Protein	%
ITP	2250	=100	1850	5060	=100
GTP	2000	89	1520	4160	82.2
UTP	420	19	350	960	18.9
CTP	250	11	245	670	13.2
ATP	120	5	140	370	7.3

[a] The activity of the enzyme on several substrates was determined with assay B at pH 8.5, 25°, and 334 nm, using appropriate dilutions of the enzyme. The molarities given by the K_M value for each substrate tested were applied: 0.1 mM GTP, 1.0 mM ITP, 10.0 mM UTP, 10.0 mM CTP, and 1.0 mM ATP. V_{max} was extrapolated from the linear parts of the Lineweaver–Burk curves and calculated for the appropriate amount of enzyme used, applying the estimated enzyme molecular weight of 52,000. In both cases the highest activity obtained with ITP was standardized at 100%.

nonspecific. In three separate preparation procedures the ratio of NTP–AMP transphosphorylase to adenylate kinase activity remained almost constant (7–9%). These findings suggest that the enzyme, purified in this way, was most likely not contaminated with adenylate kinase.

Discussion

In mitochondria, the NTP–AMP transphosphorylase is assumed to catalyze mainly in the direction of AMP phosphorylation. The ADP would be available for the oxidative phosphorylation, for the substrate level enzymes, and for further phosphorylation with adenylate kinase and NTP–NDP kinase.

In order to direct the NTP–AMP transphosphorylase reaction toward AMP phosphorylation, ADP and GDP must be removed rapidly; even small increase in these products leads to equilibrium or inhibition (K_{eq} = 0.82; Table IV; K_I(ADP); Table V). The NTP–AMP transphosphorylase itself may regulate the concentrations of ADP and GDP. *In vitro* experiments, however, favor the AMP formation. The magnesium concentration is very important for the myokinase (Noda, 1958), adenylate kinase (Markland and Wadkins, 1966; Glaze and Wadkins, 1967), and NTP–NDP kinase (Goffeau *et al.*, 1967). Similarly, the ratio nucleotide:Mg^{2+}, rather than the absolute concentration of the constituents, determine the reaction rate of the NTP–AMP transphosphorylase (Figures 5 and 6).

About 14% of the optimum enzyme activity can be detected without magnesium (Figure 5). The enzyme could have either a magnesium–nucleotide complex bound initially to it (see also Figure 3) or two binding sites, one for a magnesium–nucleotide complex and another for a nucleotide alone, thus explaining this activity. Similar observations were made with NTP–NDP kinase (Pedersen, 1968; Wålinder, 1968; Mourad and Parks, 1965) and with myokinase (Callaghan and Weber, 1955), which differs significantly in some properties from mitochondrial adenylate kinase (Markland and Wadkins, 1966). Callaghan and Weber (1955) reported that the myokinase cannot be phosphorylated at all, but seems to form an enzyme–nucleotide complex, which probably only contains AMP. Mitochondrial NTP–NDP kinase, however, forms a phosphorylated enzyme intermediate (Pedersen, 1968). Wålinder (1968) even determined the ^{32}P location in the protein molecule of erythrocytic NTP–NDP kinase.

Proposed Reaction Mechanism of the NTP–AMP Transphosphorylase. The reaction mechanism of adenylate kinase and NTP–NDP kinase are well known (Callaghan and Weber, 1955; Markland and Wadkins, 1966; Mourad and Parks, 1966; Glaze and Wadkins, 1967; Goffeau *et al.*, 1967; Su and Russell, 1968). From the results described in this paper conclusions could be drawn for the reaction mechanism of the NTP–AMP transphosphorylase. Both GTP and AMP must add to the enzyme before any product can be released. Thus a "sequential" type of reaction mechanism, according to Cleland's formulation (1963), can be proposed. The K_M values of the substrates vary (Figure 8) or remain constant (Figure 9). No family of parallel lines was obtained as proposed in a "Ping-Pong" mechanism and as shown with NTP–NDP kinase (Goffeau *et al.*, 1967; Pedersen, 1968; Wålinder, 1968).

A "Ping-Pong" mechanism can also be excluded for the reverse reaction (AMP formation). The only way the phosphate is transferred from ADP to another nucleoside diphosphate is by the specific release of AMP. A maximum velocity with ADP could never be reached (Figure 7), since the K_I for ADP is only three times higher than its K_M (Table V). The noncompetitive inhibition of ADP and GDP suggest a possible "ordered-sequential" type of reaction mechanism. This is confirmed when ADP is the only substrate and ATP is released as competitive inhibitor to AMP. This reaction is specific for adenylate kinase, but nonspecific for the NTP–AMP transphosphorylase. The K_M value of ADP (Table V) is also not comparable with those for adenylate kinase or myokinase (Markland and Wadkins, 1966). Thus the nonspecificity clearly distinguishes the NTP–AMP transphosphorylase from adenylate kinase and myokinase.

The K_I for ATP is about 50 times lower than its K_M in the

reaction with AMP and ATP. This affinity becomes explicable when the enzyme reacts with GTP or ITP and is inhibited by ATP. The low reaction rate of the enzyme with ATP (about 7%, Table VI) may constitute a bypass property. Adenylate kinase has a reaction rate approximately 70 times higher. Thus the mitochondrial ATP concentration has an important effect on the activities of these enzymes.

The NTP–AMP transphosphorylase shows its highest reaction rate with ITP (Table VI). This finding is not clearly understood. Heldt and Klingenberg (1965) stated that mitochondria do not contain ITP. However, Siekevitz and Potter (1955) observed ^{32}P incorporation into endogenous ITP in rat liver mitochondria. Heppel *et al.* (1959) showed that an inosine diphosphatase occurs in calf liver acetone powder extracts. Furthermore, IDP–ADP and IMP–AMP transaminases were reported (Deutsch and Nilsson, 1954; Mendicino and Muntz, 1958; Lee, 1957). Adenylic deaminase (Krebs and Hems, 1955) from brain mitochondria, probably bound to submitochondrial particles (Weil-Malherbe and Green, 1955), was also described. All these enzymes have very high reaction rates and their equilibrium seems to lie completely on the side of inosine nucleotide amination. This was shown by incubation experiments with either ITP, IDP, and IMP (Deutsch and Nilsson, 1954; Mendicino and Muntz, 1958). The ADP formation from inosine nucleotides may be another bypass property of the NTP–AMP transphosphorylase for these enzymes mentioned.

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