AN IMPROVED PURIFICATION AND CHARACTERIZATION OF NUCLEOSIDE TRIPHOSPHATE-ADENOSINE MONOPHOSPHATE TRANSPHOSPHORYLASE

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by

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ABSTRACT

An improved purification scheme has been devised for nucleoside triphosphate-adenosine monophosphate transphosphorylase from bovine heart mitochondria. Extraction of the enzyme from mitochondria is achieved by treatment with digitonin, extraction with phosphate buffer, and sonication. The enzyme is then purified by the following techniques: ammonium sulfate gradient solubilization on a Celite support material, Sephadex G-75 chromatography, heat denaturation, SP-Sephadex chromatography, and chromatography on Sephadex G-75 superfine. This surpasses any other previously reported purifications, yielding nearly homogeneous enzyme, 400-fold purified with a specific activity of 485 units/mg protein.

A careful investigation of the enzyme's general stability and physical properties has also been made. Nucleoside triphosphate-adenosine monophosphate transphosphorylase was found to be very stable at high temperatures and at high ionic strength. It was found to be very labile at low ionic strength, low temperatures, and dilute protein concentrations. pH-stability optima of 7.5 at 30° and 8.5 at 5° were determined. By sucrose density gradient isoelectric focusing the enzyme was shown to have an isoelectric point of 8.8 at 20°. Gel filtration and SDS gel electrophoresis indicated it to be monomeric with a molecular weight of 30,000.

Experiments involving treatment of mitochondria with digitonin were used to investigate the enzyme's intramitochondrial location. At least 24% of the total mitochondrial nucleoside triphosphate-adenosine monophosphate transphosphorylase activity was shown to be located in the outer membrane compartment with the remainder in the inner membrane region.

Evidence was presented for the possible existence of two forms of the enzyme, perhaps corresponding to the outer and inner membrane compartments. The postulated function of the inner membrane enzyme in connection with the succinyl thiokinase reaction and the analogy of the two forms with the carnitine palmityltransferase system were discussed.

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ABBREVIATIONS

A - absorbance

AMP, ADP, ATP - adenosine 5'-mono-, di- and triphosphate

CMP, CTP - cytidine 5'-mono- and triphosphate

dATP - deoxyadenosine 5'-triphosphate

dCMP, dGMP, dTMP - deoxycytidine, deoxyguanosine, and deoxythymidine 5'-monophosphates

dHMP - 5'-hydroxymethyl-deoxycytidine monophosphate

DNA - deoxyribonucleic acid

EDTA - ethylenediaminetetraacetic acid

GMP, GDP, GTP - guanosine 5'-mono-, di- and triphosphate

ITP - inosine 5'-triphosphate

K_{av} - partition coefficient

NADH - nicotinamide adenine dinucleotide, reduced form

NMP, NTP - any nucleoside mono or triphosphate

NTP-AMP tpp - Nucleoside triphosphate-adenosine monophosphate transphosphorylase

pI - isoelectric point

RNA - ribonucleic acid

SDS - sodium dodecyl sulfate

Tris - tris(hydroxymethyl)aminomethane

UTP - uridine 5'-triphosphate

XMP - any nucleoside monophosphate

I. INTRODUCTION

In mitochondria the class of enzymes known as nucleoside monophosphokinases, together with the nucleoside diphosphokinases, facilitate the distribution of high energy phosphoryl groups derived from substrate-level and oxidative phosphorylation throughout the three levels of phosphorylation in the free nucleotide pool:

$$\text{NUCLEOSIDE MONOPHOSPHATES} \xrightarrow{\text{monophospho-kinases}} \text{NUCLEOSIDE DIPHOSPHATES} \xrightarrow{\text{diphospho-kinases}} \text{NUCLEOSIDE TRIPHOSPHATES}$$

In doing so these enzymes make a major contribution to the synthesis of nucleoside triphosphates and in turn RNA, DNA, and coenzymes.

Whereas a great deal is known about the regulation of energy distribution amongst the adenine nucleotides mediated by adenylate kinase, much less is known about the control of distribution amongst the other nucleotides. It is in this area that the nucleoside monophosphokinase, nucleoside triphosphate-adenosine monophosphate transphosphorylase*, may play an important role. Previous investigations of this enzyme have been hampered by ineffective purification attempts, and a failure to obtain homogeneous enzyme. Thus, relatively little is known about this enzyme's properties or function, although it is thought to be directly connected with the succinyl thiokinase reaction.

The purpose of this thesis then was to improve upon the previously reported purification schemes for NTP-AMP tpp in hopes of obtaining from bovine heart mitochondria, homogeneous enzyme in high yields. At the same time, careful investigations of the enzyme's general stability and

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* to be abbreviated as NTP-AMP tpp

physical properties were to be made. It was hoped that this research would eventually lead to a better understanding of the enzyme's function and its relationship to other nucleoside monophosphokinases and nucleoside diphosphokinase in the control of energy distribution amongst nucleotides in mitochondria.

II. LITERATURE REVIEW

The general class of enzymes known as the nucleoside monophosphokinases catalyze the reversible transfer of a phosphate from a nucleoside triphosphate to a nucleoside monophosphate, forming a new phosphoanhydride bond*:

$$NTP + XMP \rightleftharpoons NDP + XDP$$

As early as 1943 (5), adenylate kinase, an enzyme belonging to this group, was extensively purified. It was not until the mid-fifties, however, when enzymes catalyzing the transfer between nucleotides other than just adenine nucleotides were simultaneously discovered, that the general class of enzymes known as nucleoside monophosphate kinases was recognized (6-11). Since then extensive work on the identification, purification, and characterization of the various enzymes belonging to this group has been carried out.

In 1959 Strominger, Heppel, and Maxwell (12,13) discovered two distinct categories of nucleoside monophosphokinases: (a) those enzymes catalyzing transphorylation between AMP and nucleoside triphosphates and (b) those catalyzing transphorylation between nucleoside monophosphates and ATP. All the nucleoside monophosphokinases subsequently investigated have fallen into one of these two classes. Whereas the first category has been found to comprise only one enzyme with a broad substrate specificity, the second category contains several enzymes exhibiting very narrow specificities.

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* for general reviews on the monophosphokinases see references (1-4)

A. NTP-AMP Transphosphorylase

Although variously credited with different names and specificities, a single enzyme which is nonspecific for the nucleoside triphosphate catalyzes the transfer of phosphate from a nucleoside triphosphate to AMP. It has become known as NTP-AMP tpp.

Heppel (13) was the first to study this enzyme, purifying it 4-fold from calf liver acetone powder and separating it from the ATP-NMP nucleoside monophosphokinases. It was shown to catalyze phosphorylation of AMP by ITP, UTP, GTP, CTP, and ATP. No other nucleoside monophosphate could replace AMP.

In 1961 Chiga et al. (14) investigated an enzyme they called 6-oxypurine nucleoside triphosphate-adenosine monophosphate transphosphorylase which they partially purified from swine liver. This enzyme appeared to have a narrower specificity towards the nucleoside triphosphate than that of Heppel, utilizing only GTP or ITP as phosphate donors. In view of this they suggested Heppel had perhaps isolated several enzymes.

More recently work has appeared in the literature on a GTP-AMP phosphotransferase, again the same enzyme but so-called because of its highest specificity for GTP. Heldt and Schwalbach (15) were the first to investigate the enzyme's subcellular location, showing it to be localized in the mitochondria of rat liver. They also performed labelling experiments in which they showed that $[^{32}P]$ ADP rather than $[^{32}P]$ ATP is the main product of the succinyl thiokinase reaction*. This indicated a possible function of GTP-AMP phosphotransferase in connection with substrate level phosphorylation, specifically in the regeneration of GDP

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* for reaction see p. 73

for the succinyl thiokinase reaction.

Lima and Vignais (16) simultaneously carried out similar experiments with regard to the rat liver enzyme's function and obtained results which agreed with those of Heldt and Schwalbach. They determined that 70% of GTP-AMP phosphotransferase and most of succinyl thiokinase were linked to the inner membrane of mitochondria. That the two enzymes were located in the same mitochondrial compartment further supported their connection.

The most recent and extensive investigation of NTP-AMP tpp has been made by Albrecht (17) on the bovine heart mitochondrial enzyme. He succeeded in purifying the enzyme 40-fold, using a purification scheme involving: extraction by sonication, ammonium sulfate fractionations, DEAE-cellulose chromatography and heat denaturation. His purest enzyme preparation had a final specific activity of 136 units/mg and was estimated from gel electrophoretic patterns to be approximately 75% pure. Using numerous other techniques including ion-exchange chromatography, gel filtration, calcium phosphate or aluminum hydroxide gels, Albrecht failed to further purify the enzyme. Crystallization attempts were also unsuccessful.

Albrecht also investigated a number of the properties of his enzyme preparation. He found NTP-AMP tpp to be stabilized by the presence of ions but unstable when exposed to freezing, dilute solutions or low ionic strength. The enzyme showed activity over a broad pH range with an optimum at 8.5. Albrecht estimated its molecular weight to be 52,000 from Sephadex G-200 gel filtration.

Kinetic studies performed on the enzyme confirmed its nonspecificity

with respect to the nucleoside triphosphate. Albrecht determined the following Km's for the NTP's:

GTP	5.6×10^{-5} M
ITP	6.3×10^{-4} M
ATP	1.0×10^{-3} M
UTP	7.4×10^{-3} M
CTP	9.1×10^{-3} M

No other nucleoside monophosphates could substitute for AMP as the phosphate acceptor. NTP-AMP tpp activity was found to be stimulated by divalent cations, optimally by 4.0×10^{-3} M Mg^{+2}.

From GTP labelling experiments Albrecht concluded the enzyme followed a mechanism in which a nucleotide-enzyme complex and not a phospho-enzyme complex is formed.

B. ATP-NMP Transphosphorylases

ATP-NMP reactions are catalyzed by several enzymes each of which is very specific for a certain nucleoside monophosphate. Contrary to NTP-AMP tpp, the investigations of the ATP-NMP tpp's have been quite extensive and a great deal is known about their properties.

1. Adenylate Kinase

Of the ATP-NMP transphosphorylases, adenylate kinase or properly ATP-AMP phosphotransferase, is the most extensively studied*. First purified from rabbit muscle in 1943 by Colowick and Kalckar (5), it has been subsequently purified from a wide variety of sources some of which include: swine liver (19), bovine liver mitochondria (20), rat liver (21,22),

* for a general review see (18)

porcine muscle (23), human muscle (24), baker's yeast (25), cockroach muscle mitochondria (26), bovine eye lens (27), Bacillus subtilis (28), and E. coli (29).

As the major function of adenylate kinase is believed to be the distribution of high energy among the adenine nucleotides, it is abundant where the turnover of energy is great: muscle and mitochondria. Brdiczka et al. (30) have shown that the rat liver mitochondrial enzyme is located between the outer and inner membranes. However, not all cellular adenylate kinase appears to be confined to mitochondria. Criss (31) has reported the cellular distribution of 4 isoenzymes (designated I, II, III, and IV in order of increasing pI's) from rat liver. The minor isoenzyme I, and III were found in the nuclei; III, the predominant isoenzyme, was located in the mitochondria, and the cytosol contained isoenzyme II.

The muscle and liver enzymes seem to differ somewhat in their physical properties. The rabbit muscle enzyme contains free -SH groups which when reacted with sulfhydryl reagents, result in loss of activity (32). It is thought that the -SH groups are not involved in substrate binding but rather are necessary for the maintainance of a proper conformational state. The bovine liver enzyme (20), on the contrary, has no free -SH groups and is inhibited by disulfide reducing agents. The liver enzymes have isoelectric points around 7.5 whereas that of the rabbit muscle enzyme has been determined to be approximately 9.3 from the amino acid composition (33) or 10.6 from cellulose acetate electrophoresis (34).

The majority of adenylate kinases have molecular weights of about 21,500. The yeast and rat liver enzymes have considerably higher

molecular weights, however. The molecular weight of the enzyme from yeast is estimated to be 41,000 (25). The rat liver III enzyme shows a tendency to aggregate, existing in monomer, dimer, or trimer forms (23,000, 46,000, and 68,000 in molecular weight respectively) dependent on the protein concentration (21). The rat liver II isoenzyme appears to have a molecular weight of 46,000-49,000.

Adenylate kinase requires a bivalent metal for activity, catalyzing the transfer of phosphate from a Mg-ATP complex to AMP:

$$MgATP + AMP \rightleftharpoons MgADP + ADP$$

It is very specific for its acceptor, using only AMP and to a lesser degree, dAMP. It is somewhat less specific for the nucleoside triphosphate. For instance the rabbit muscle enzyme can use 2'-dATP, CTP, GTP, UTP, and ITP as well as ATP, although much less effectively (35)

Considerable investigation into the mechanism of adenylate kinase has been carried out. The enzyme appears to have two binding sites--one for the Mg^{+2} chelated nucleotide and a second for the unchelated nucleotide. Rhoads and Lowenstein (36) have concluded from their equilibrium isotope exchange and initial velocity studies that the rabbit muscle enzyme follows a random Bi-Bi mechanism. This appears to be the case for the yeast enzyme as well (37). However, Markland and Wadkins (38) have tentatively suggested an ordered Bi-Bi mechanism for the bovine liver adenylate kinase; while recently Brownson and Spencer (39) have found data consistent with a rapid equilibrium random mechanism for a human erythrocyte enzyme.

2. GMP Kinase

ATP-GMP phosphotransferase, commonly known as guanylate kinase, has been studied and partially purified from several sources: hog brain (40), E. coli extracts (41), human erythrocytes (42), Sarcoma ascites cells (43,44), rat liver (47), and Ascaris lumbricoidis (48). A deoxyguanylate kinase shown to be identical to the GMP enzyme has also been isolated from calf thymus (45). The reaction catalyzed by this enzyme is:

$$(d)ATP + (d)GMP \rightleftharpoons (d)ADP + (d)GDP$$

It is highly specific with respect to both the phosphate acceptor and phosphate donor. The nucleoside monosphosphate binding site is selective for the guanine moiety, the enzyme being active only with GMP, dGMP, and 8 aza GMP (40,41,42,45). 6-thio GMP is inactive as a substrate but serves as a competitive inhibitor of GMP (42). IMP was also found to serve as a substrate but at a rate much lower than that of the guanosine compounds (41,42).

ATP and dATP appear to be the only natural phosphate donors for the enzyme (41), although very low activities with CTP, ITP, and UTP have been observed (40).

The existence of isoenzymes of guanylate kinase has been demonstrated in human erythrocytes and Sarcoma 180 ascites cells (43). These isoenzymes have isoelectric points in the vicinity of 5.0 and molecular weights ranging from 18,500 to 24,000.

Although no definite mechanism for guanylate kinase has been established as yet, Miech et al. (40), studying the hog brain enzyme, have found no indication of a phosphorylated enzyme intermediate.

Initial velocity studies performed by Agarwal and coworkers on the erythrocyte enzyme (42) showed that no product was released from the enzyme surface between addition of ATP and GMP, thus eliminating a ping-pong mechanism.

This monophosphokinase like others requires a divalent cation such as Mg^{+2} or Mn^{+2} for optimal activity. It has also been discovered that guanylate kinase activity is markedly stimulated by monovalent alkali metal ions, especially K^+, and by NH_4^+ (40,41,45,46). Kinetic analysis (41) suggests, however, that the mechanism of activation is probably different for K^+ than for NH_4^+.

3. dTMP Kinase

Thymidylate kinase catalyzes the following reaction:

$$dTMP + (d)ATP \rightleftharpoons dTDP + (d)ADP$$

It has been partially purified from E. coli (49,50,51), L cells (52), Ehrlich Ascites carcinoma (53), Landschutz Ascites cells (54), and calf thymus (51,55). More extensive purification and investigation of the enzymes from E. coli and mouse ascites hepatoma have been carried out by Nelson and Carter (56) and Kielley (57), respectively.

The mammalian and bacterial enzymes appear to be similar in their specificity. Only dTMP and to a much lesser extent, dUMP, show activity as phosphate acceptors while only ATP or dATP appear to be effective donors. Both enzymes show the usual requirement for Mg^{+2}.

Whereas the E. coli enzyme is relatively stable, the enzymes from mouse hepatoma and calf thymus are extremely labile in nature, requiring the presence of both a thiol compound and substrate for

stabilization. These enzymes also appear to differ in molecular weight, 65,000 being reported for the E. coli thymidylate kinase (51) and 35,000 for the mouse hepatoma enzyme.

An interesting feature of the mouse hepatoma enzyme is its extraordinary inhibition by ADP (57). It has been suggested that the enzyme in mitochondria may exist in an inactivated state due to a high concentration of ADP. This would explain experiments in which activation of dTMP kinase has been produced by disruption of mitochondria (58).

4. CMP-dCMP-UMP Kinase

It appears that this enzyme catalyzes the phosphorylation of CMP and dCMP. However, conflicting evidence has been presented as to whether it is also capable of phosphorylating UMP.

An enzyme catalyzing the transfer of phosphate from ATP to CMP or dCMP (UMP not tried) was first partially purified from Azotobacter vinelandii (59).

In 1966 Sugino, et al (55) partially purified from calf thymus, a dCMP kinase. They found that surprisingly this enzyme could employ as a phosphate acceptor not only dCMP and CMP but also UMP. ATP and dATP, 80% as effectively, were found to serve as phosphate donors. The dCMP kinase activity was shown to have an absolute requirement for thiols.

In 1969, Ruffner and Anderson (60) purified a similar enzyme, UMP kinase, 300-fold from Tetrahymena pyriformis. It could also catalyze the phosphorylation of CMP and dCMP. As with the calf thymus enzyme only dATP apart from ATP could serve as a donor, but only 1/10 th as

effectively. This enzyme, however, did not require the presence of thiol compounds.

Initial velocity and isotope exchange experiments (61) have indicated that this Tetrahymena pyriformis enzyme proceeds by a sequential mechanism. It has been suggested that there may be two binding sites on the enzyme for UMP and that CMP binds only to one of them (60).

Recently another dCMP kinase has been extensively purified from rat liver by Maness and Orengo (62). With ATP and dATP as donors it can also use CMP, dCMP, and UMP as acceptors. When CMP is used, however, all nucleoside triphosphates can act as donors to a significant extent. This enzyme like the other mammalian one requires thiol compounds for activation. It has a low molecular weight of about 15,000 and shows a tendency to aggregate (63).

Whereas the calf thymus, rat liver, and Tetrahymena pyriformis enzymes utilize UMP as a substrate, enzymes from other sources seem capable of phosphorylating only dCMP and CMP. Hiraga and Sugino (64) reported that they were able to separate dCMP-CMP and UMP kinases from E. coli extracts as two distinct entities on DEAE cellulose chromatography. Mutants of Salmonella typhimurium have been isolated which carry mutations in the structural gene coding for UMP kinase (65). CMP kinase activity, however, was not affected, indicating the presence in this case of two different enzymes for phosphorylating CMP and UMP.

5. Deoxyribonucleoside Monophosphokinases

Except in the special case of phage infected E. coli, it appears that deoxyribonucleotides are phosphorylated by individual kinases with specificity based on the base and not the sugar constituent. For the

deoxyribonucleotide monophosphates of adenine, cytosine, and guanine this means phosphorylation of each is accomplished by the corresponding ribonucleotide monophosphokinase. Thymidylate and perhaps deoxyuridylate are phosphorylated by thymidylate kinase.

Two specific deoxynucleoside monophosphate kinases, however, have been discovered in extracts of bacteriophage infected E. coli. T2 and T4 phage infection of E. coli resulted in the appearance of dHMP kinase activity (DNA of T even phages contain 5-hydroxymethyl-deoxycytidylate instead of deoxycytidylate) and an increase in dGMP and dTMP activities (66,67,68). It was found that these activities, which could be separated from the normal E. coli kinases, in fact, resided in one protein. This partially purified dHMP-dGMP-dTMP kinase showed a little activity toward dUMP but none towards dAMP or ribonucleotides. It used only ATP or dATP as a phosphoryl donor.

Similarly infection of E. coli with a T5 phage produced an enzyme capable of phosphorylating four deoxynucleotide monophosphates: dTMP, dGMP, dCMP, and dAMP (69). Again only ATP or dATP could serve as phosphate donors and ribonucleoside monophosphates were not phosphorylated.

These are the only two enzymes,so far, which can phosphorylate specifically only deoxyribonucleotides and which are capable of using more than one type of deoxyribonucleoside monophosphate as a substrate.

III. EXPERIMENTAL

A. Materials

Acrylamide - Bio-rad laboratories, Eastman Kodak

Adenosine 5' monophosphate (sodium salt, type II) - Sigma Chemical Co.

β-Alanine - Eastman

Ammonium persulfate - Bio-rad

Ammonium sulfate (analytical grade) - J.T. Baker Chemical Co.

Ampholine (40% pH 3-10, 40% pH 8-10, and 20% pH 9-11) - LKB Products

Blue dextran - Pharmacia

Bovine serum albumin - Sigma

Bromophenol blue - J.T. Baker

Celite 545 - Fisher

α-Chymotrypsinogen-A (bovine pancreas, type II) - Sigma

Coomassie Brilliant Blue R - Sigma

Creatine phosphokinase (rabbit muscle) - Sigma

Cytochrome c (horse heart, type VI) - Sigma

Deoxycholate (sodium salt) - Sigma

Digitonin (80%) - Sigma

Ethanolamine - J.T. Baker

Ethylenedinitrilotetraacetic acid (disodium salt, reagent grade) - Matheson Coleman and Bell

Glycerol (spectroquality) - Matheson Coleman and Bell

Guanosine 5' triphosphate (sodium salt, type III) - Sigma

Lactate dehydrogenase (beef muscle, type X) - Sigma

β Lactoglobulin - Sigma

Magnesium acetate - J.T. Baker

2-Mercaptoethanol - Eastman

N,N'-Methylene-bis-acrylamide - Bio-rad

Myoglobin (equine skeletal muscle) - Sigma

NADH (disodium salt, from yeast, grade III) - Sigma

Ovalbumin (grade V) - Sigma

Phosphoenol pyruvate (mono and tricyclohexylamine salt forms) - Sigma

Phosphoric acid (reagent) - J.T. Baker

Potassium chloride (reagent grade) - Matheson Coleman and Bell

Potassium hydroxide - Matheson Coleman and Bell

Potassium phosphate (mono and dibasic, reagent) - J.T. Baker

Pyruvate kinase (rabbit muscle, types II and III) - Sigma

Riboflavin - Sigma

Ribonuclease A (bovine pancreas, type 1-A) - Sigma

Sephadex G-25, G-75, and G-75 superfine - Pharmacia

Sodium azide - Fisher

Sodium chloride (reagent grade) - Matheson Coleman and Bell

Sodium dodecyl sulfate - Sigma

Sodium phosphate (mono and dibasic, reagent grade) - Matheson Coleman and Bell

SP-Sephadex C-50 - Pharmacia

Sucrose (reagent) - Matheson Coleman and Bell

Tert-amyl alcohol (reagent grade) - Fisher

N,N,N',N'-Tetramethylethylenediamine (electrophoresis grade) - Bio-rad

Trichloroacetic acid (reagent) - J.T. Baker

Triethanolamine hydrochloride - Sigma

Tris(hydroxymethyl)aminomethane (Sigma 7-9) - Sigma

Triton X-100 - Sigma

B. Methods

1. Assay of NTP-AMP tpp

NTP-AMP tpp was assayed spectrophotometrically by coupling it to the pyruvate kinase and lactate dehydrogenase reactions as shown below:

$$GTP + AMP \longrightarrow GDP + ADP$$

$$GDP \text{ or } ADP + \text{phospho-enolpyruvate} \xrightarrow{\text{Pyr. Kinase}} \text{pyruvate} + GTP \text{ or } ATP$$

$$\text{pyruvate} + NADH + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

The reaction was monitored by following the decrease in absorption at 340 nm due to the oxidation of NADH, using a Beckman DB-G spectrophotometer linked to a Beckman 10 in recorder. One unit of NTP-AMP tpp is defined as the amount of enzyme utilizing one µmole of GTP per min under the conditions defined below. As pyruvate kinase reacts almost as effectively with GDP as ADP, one µmole of NTP-AMP tpp activity was attributed to the oxidation of two µmoles of NADH.

For a total volume of 3 ml, the assay mixture contained 3.4 µmoles of GTP, 4.9 µmoles of AMP, 7.4 µmoles of phosphoenolpyruvate, 0.65 µmoles of NADH, 4.5 units of pyruvate kinase, 3.0 units of lactate dehydrogenase, and 1.5 ml of a stock solution (pH 8.5) containing 0.1 M triethanolamine hydrochloride, 0.008 M magnesium acetate, 0.15 M KCl, and 0.002 M EDTA. The assay medium minus the NTP-AMP tpp was incubated in the cuvette in the thermostatically controlled [30°] spectrophotometer compartment for about 5 min; after which time the appropriate enzyme aliquot was added and the absorption followed.

Figure 1 shows the region of linearity for this assay. NTP-AMP tpp activity was proportional to the rate of change in absorbance at 340 nm up to a decrease of approximately 0.35 absorbance units per min.

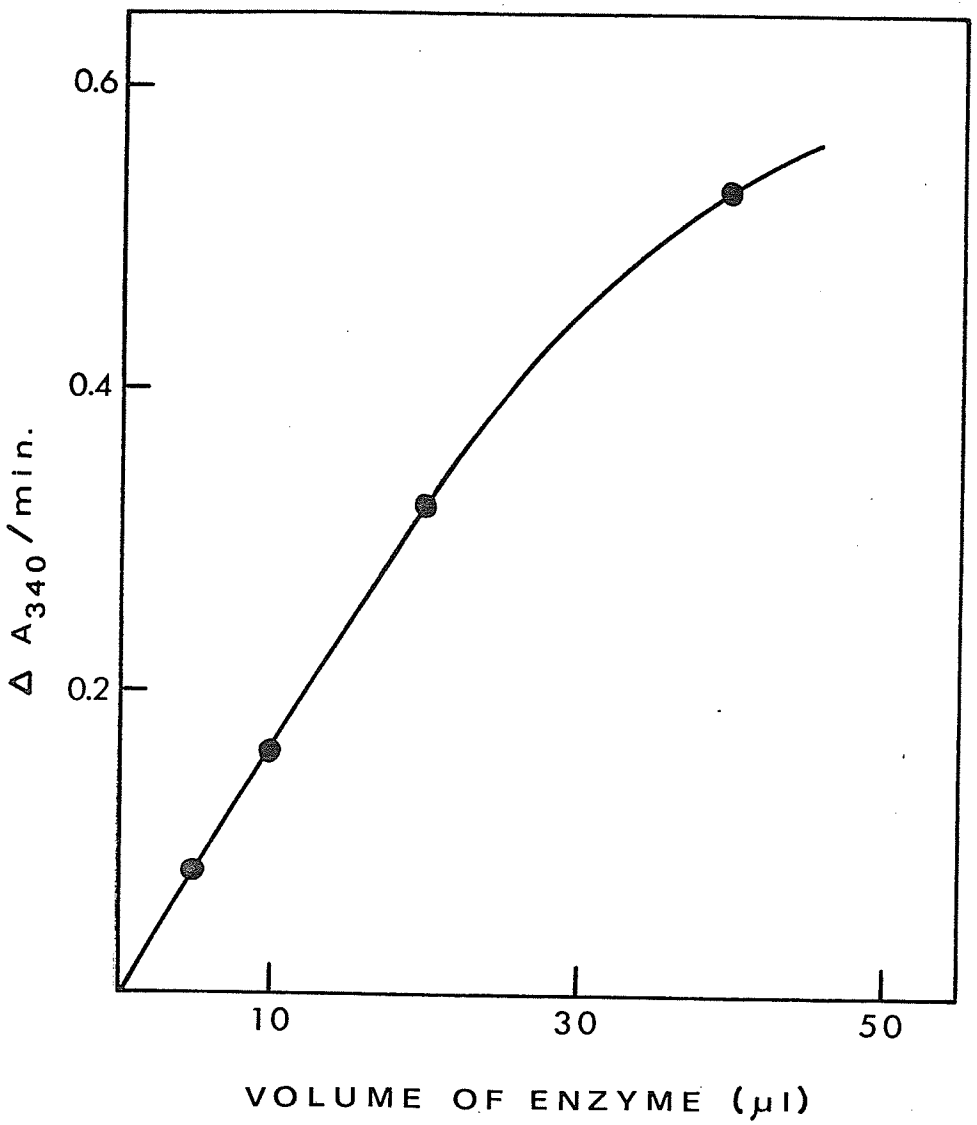

Figure 1. Region of linearity for the coupled enzyme assay for NTP-AMP tpp. The enzyme solution used had an activity of 3.89 units/ml.

2. Protein Determinations

Mitochondrial protein concentrations were determined by the Biuret method of Jacobs et al.(70) in which the protein and phospholipids are first solubilized with deoxycholate. The Biuret method (71) was also used for samples of high protein concentration (at least 1mg/ml). In this case the protein was first precipitated with trichloroacetic acid. Bovine serum albumin was used as a standard for both these methods. The protein concentration of more dilute solutions was calculated from the absorbance at 280 nm as described by Warburg and Christian (72).

3. $(NH_4)_2SO_4$ Saturation

The ammonium sulfate required for a given saturation was determined from the formula described by Noda and Kuby (73):

$$w = \frac{0.515V(S_2-S_1)}{1-S_2(0.272)}$$

where w equals the weight of $(NH_4)_2SO_4$ in g.
V is the volume of the solution at saturation S_1
and S_2 is the desired saturation*.

4. Preparation of Mitochondria

Two fresh bovine hearts obtained from the local slaughterhouse were used in each preparation of mitochondria. After removal of fat and connective tissue, the hearts were cubed and ground in a meat grinder.

The ground tissue was diluted 1:10 with cold isotonic sucrose buffer, pH 7.4, containing 0.3 M sucrose, 0.01 M triethanolamine hydrochloride, and 0.001 M EDTA and then homogenized for 15 sec. in a 1 gal. Waring Blender at "high" speed. The pH was readjusted to 7.4 with 2 N KOH and

* all $(NH_4)_2SO_4$ concentrations are given in % saturation

homogenization repeated.

Cell debris was removed by centrifugation at 500 x g for 15 min at 0° followed by filtration of the supernatant through cheese cloth. The mitochondria were then sedimented by centrifugation at 23,500 x g for 10 min at 0° and washed twice with the sucrose buffer.

5. Extraction of NTP-AMP tpp

The enzyme was usually extracted from the mitochondria by treatment with digitonin followed by an overnight PO_4^{-3} extraction and finally disruption by sonication.

a) Digitonin Treatment

The mitochondria were treated with digitonin according to the method of Schnaitman et al.(74).

Mitochondria were diluted slightly with isotonic sucrose buffer and the volume and protein concentration of the solution determined. The total mitochondrial protein was then calculated and used to determine the amount of digitonin required for a ratio of digitonin to mitochondrial protein of 1.5 mg to 10 mg.

The required quantity of digitonin was then dissolved in a volume of hot sucrose buffer approximately equal to that of the mitochondrial solution. Further heating was sometimes necessary to completely solubilize the digitonin. The digitonin solution was cooled and added gradually, with stirring, to the mitochondrial cooled in ice. Following addition, the mixture was incubated without stirring for 40 min at 5°.

The solution was then centrifuged for 30 min at 23,500 x g and 0°. The supernatant or digitonin extract was immediately made 75% in $(NH_4)_2SO_4$

and readjusted to pH 7.5 with 2 N KOH.

b) Phosphate Extraction

The disrupted mitochondria obtained from digitonin treatment were next extracted overnight in 0.075 M sodium phosphate buffer, pH 7.5. The mitochondria were resuspended in the buffer using a Potter homogenizer and brought to a total volume approximately 6 times that of the intact, packed mitochondria. The suspension was then slowly stirred overnight at 5°. In the morning it was centrifuged (23,000 x g for 30 min.) and the supernatant or phosphate extract was made 75% in $(NH_4)_2SO_4$ with readjustment to pH 7.5.

c) Sonication

The extracted mitochondria were then further disrupted by sonication or on occasion by disintegration using a Polytron apparatus.

For sonication the mitochondria were diluted with 0.075 M PO_4^{-3} buffer, pH 7.5, to an approximate protein concentration of 20 to 25 mg/ml (using the original mitochondrial protein determination). 75 ml portions were then sonicated, with cooling, a total of 5 min. in 30 sec. intervals at the maximum output of a Bronwill Biosonik IV sonicator.

When high speed rotary disintegration was used, the mitochondria were resuspended in PO_4^{-3} buffer to a total volume of about 480 ml. 25 ml portions were then disintegrated for 5, 30 sec intervals.

The sonicated or disintegrated material was centrifuged at 125,000 x g* for 60 min. at 0° to remove any remaining particles. As with the other extracts the supernatant was made 75% in $(NH_4)_2SO_4$.

The three extracts were stored separately at 5° until just before use.

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* g, here, refers to $g_{aver.}$ whereas others, unless indicated otherwise, refer to g_{max}.

6. Purification

The following purification steps were all performed at room temperature and to prevent bacterial growth buffers contained 0.02% sodium azide.

a) Ammonium Sulfate Gradient Solubilization

The first step in the purification of NTP-AMP tpp involved a technique described by King (75). In it proteins are precipitated onto Celite, a solid support material, and then resolubilized with a decreasing salt gradient.

Just prior to the Celite chromatography, the three NTP-AMP tpp extracts were combined and centrifuged (17,400 x g for 30 min. at 0°). Usually the entire preparation was used. However, in cases where the protein greatly exceeded 2000 mg, two columns were run.

The precipitated protein was dissolved in 800 ml of 0.05 M Tris-Cl buffer, pH 8.0, containing 147 g of Celite. To this was added, with stirring, sufficient $(NH_4)_2SO_4$ to bring the solution to 80% saturation. The mixture was stirred for about 1 hr. and then packed into a column (2.5 x 100 cm.) under 3 psi nitrogen pressure. The column was eluted with a linear gradient decreasing from 80 to 0% $(NH_4)_2SO_4$ saturation in 0.05 M Tris-Cl, pH 8.0 (2,000 ml of 0% and 1660 ml of 80% $(NH_4)_2SO_4$). The $(NH_4)_2SO_4$ saturation of the fractions collected was determined from conductivity measurements after 40-fold dilutions, using a Radiometer Type CDC 2e conductivity meter. The NTP-AMP tpp activity was located, pooled, and brought to 75% $(NH_4)_2SO_4$ in order to precipitate the protein.

b) Sephadex G-75 Chromatography

The enzyme was next fractionated using gel filtration. The

precipitated protein was centrifuged (17,400 x g for 30 min.) and redissolved in a small volume of 0.1 M Tris-Cl buffer, pH 7.5. This was applied to a column (5.0 x 100 cm.) of Sephadex G-75 equilibrated with the same buffer and eluted. The enzyme activity was found, combined, and then concentrated to a protein concentration of at least 1 mg/ml using a series of Amicon ultrafiltration cells fitted with Diaflo PM-10 membranes. The concentrated sample was stored at 75% $(NH_4)_2SO_4$.

c) Heat Treatment

Prior to heat treatment the enzyme was centrifuged (17,400 x g for 30 min.) and the precipitate redissolved in a volume of 0.1M Tris-Cl buffer, pH 7.5, giving a protein concentration of approximately 2 mg/ml. This solution was made 35% in $(NH_4)_2SO_4$ to stabilize the NTP-AMP tpp and then heated for 3 min. in a 65° water bath. The denatured protein was removed by centrifugation (27,000 x g for 30 min.) and the supernatant made 75% $(NH_4)_2SO_4$.

d) SP-Sephadex Chromatography

A volume of heat treated sample containing approximately 600 units of NTP-AMP tpp activity was centrifuged as usual and the pellet dissolved in 0.1 M Tris-PO_4 buffer, pH 6.0. This was immediately applied to a column (2.5 x 100 cm.) of SP-Sephadex C 50 equilibrated with the same buffer. The SP-Sephadex was prepared by incubating it in 0.5 M Tris-PO_4, pH 6.0 (usually overnight) and washing it extensively on a sintered glass funnel with 0.1 M Tris-PO_4 pH 6.0. The column was eluted with a linear gradient increasing from 0 to 0.5 M NaCl in 0.1 M Tris-PO_4 pH 6.0 (total gradient volume was 2425 ml). The enzyme containing fractions were pooled, titrated to pH 7.5 and then concentrated to a volume

less than 1 ml using Amicon cells. The enzyme solution was stored in 75% $(NH_4)_2SO_4$.

e) Sephadex G-75 Superfine Chromatography

Several samples after SP-Sephadex were combined, centrifuged and redissolved in 0.1 M Tris-Cl pH 7.5. This was then chromatographed on a Sephadex G-75 superfine column (2.5 x 100 cm.) equilibrated in the same buffer. The purified enzyme was concentrated down to less than 1 ml and stored in 75% $(NH_4)_2SO_4$.

7. Analytical Techniques

a) SDS Gel Electrophoresis

The standard procedure of Weber and Osborn (76) was used for SDS gel electrophoresis. An ammonium persulfate solution of 8 mg/ml rather than 15 mg/ml was used, however. Sample loads of approximately 0.01 mg were applied. The gels were electrophoresed at a constant current of 8 ma/tube, using bromophenol blue as a tracking dye. The protein was stained for 1 hr. using the 0.25% Coomassie Blue solution described by Weber and Osborn and destained in a Bio-rad Model 170 diffusion destainer.

b) Gel Isoelectric focusing

Gel electrofocusing was performed according to the method described by Wrigley (77). Ammonium persulfate was used most often to catalyze polymerization of the gels. In some cases 1 mM mercaptoethanol was also incorporated into the gels to prevent possible artifact formation as a result of oxidative side products. The protein sample (about 0.1 mg) was usually distributed throughout the gel. pH 3-10 ampholytes were used to form the pH gradient. 0.4% ethanolamine and 0.2% phosphoric acid

were used as the cathodic and anodic buffers, respectively. Electrofocusing was performed in a Buchler apparatus cooled by running water. A constant voltage of 150 V was maintained. A gel containing cytochrome c was used to monitor the progress of the isoelectric focusing. Usually the runs were terminated about an hour after cytochrome c had focused which meant a total electrofocusing time of about 4-5 hours.

The gels were stained for 1 hr. in either:

(1) a bromophenol blue stain formulated by Awdeh (80) containing 0.2% bromophenol blue in ethanol:water:acetic acid (50:45:5) and destained with ethanol:water:acetic acid (30:65:5)

or (2) 0.1% Coomassie blue in water:ethanol:acetic acid (50:45:5) and destained in water:ethanol:acetic acid (50:40:5) (81).

c) Cathodic Disc Gel Electrophoresis-pH 4.3 System

Cathodic disc electrophoresis was carried out using a slight modification of the Reisfield, Lewis, and Williams system (82), as described in a Canalco booklet (83). In this system proteins stack at pH 5.0 and run at a pH of 4.3. The final acrylamide concentration of the separating gel is 7%.

Protein loads of approximately 0.1 mg were used. The gels were electrophoresed at 5 ma/tube, using cytochrome c as a tracking dye. They were then stained for 1 hr. in a 1% (w/v) Amido Schwartz solution in 7% acetic acid and destained with 7% acetic acid.

8. Characterization

a) Total Content of the Enzyme in Mitochondria

Mitochondria were treated with the following reagents in an attempt to determine the total NTP-AMP tpp content.

(i) tert-Amyl Alcohol

Mitochondria were solubilized with t-amyl alcohol using the procedure outlined by Griffiths and Wharton (84). They were first diluted with a volume of a 0.9% KCl solution equal to 1/3 their volume. t-amyl alcohol (1/9th of the volume of mitochondrial suspension) was then added dropwise, with stirring, to the cooled mitochondria. After addition the mixture was rapidly warmed to 20° by immersion in a 60° water bath and maintained at 20° for 10 min. This was followed by cooling in a salt-ice bath and then centrifugation at 100,000 x g ($g_{aver.}$) for 1hr. to remove any insoluble material.

(ii) Triton X-100

Triton X-100 was used basically as described by Jacobs and Andrews (85). Mitochondria were suspended in PO_4^{-3} buffer to give a protein concentration of approximately 25 mg/ml. With vigorous stirring .003 ml of a 20% (v/v) Triton X-100 solution for every mg of protein was added to the cooled mitochondrial solution. The mixture was incubated at 0° for 2 hr. and then centrifuged as previously described.

(iii) Deoxycholate-KCl

The method of Burkhard and Kropf (86) was used to treat mitochondria with deoxycholate-KCl. 5 ml of a mitochondrial solution (35 mg/ml) was suspended in 20 ml of a 0.02 M deoxycholate-10% KCl solution, pH 7.8. The insoluble material was removed by centrifugation as described above.

b) Molecular Weight

(i) Gel Filtration

The molecular weight of NTP-AMP tpp was determined by gel filtration according to the technique of Laurent and Killander (87).

An enzyme sample and marker proteins of known molecular weight were chromatographed on a Sephadex G-75 column (2.5 x 100 cm.) and their elution volumes determined. Blue dextran was used in each run to find the void volume. The logs of the molecular weights of the markers were then plotted versus their partition coefficients (K_{av}) calculated as shown below.

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

where Ve is the elution volume
Vt is the bed volume
and Vo is the void volume.

Using the partition coefficient obtained for NTP-AMP tpp, the molecular weight was interpolated from the calibration curve.

(ii) SDS Gel Electrophoresis

The subunit molecular weight of NTP-AMP tpp was obtained from SDS gel electrophoresis as described by Weber and Osborn (76).

c) Isoelectric Point

(i) Gel Isoelectric Focusing

The enzyme's isoelectric point was found from gel isoelectric focusing, using the same basic procedure previously described. The enzyme and proteins of known isoelectric point were electrofocused simultaneously. A calibration curve of the mobilities (distance of the band ÷ total length of the gel) of the markers versus their isoelectric points was made and

used to determine unknown pI's.

(ii) Density Gradient Isoelectric Focusing

The isoelectric point of NTP-AMP tpp was also determined by isoelectric focusing in a sucrose density gradient. Preliminary experiments had indicated the enzyme's isoelectric point to be approximately 9.3 (at 4°). Therefore, to determine an accurate pI, isoelectric focusing was repeated using a gradient ranging from pH 8-11.

Basically the procedure used was that described by the LKB manual (88). A total of 0.5% pH 8-10, 0.5% pH 9-11, and 0.1% pH 3-10 ampholytes were used to form the pH gradient. The electrofocusing was carried out in an LKB 8101 apparatus, thermostatically controlled at 20°. The voltage was gradually increased until 400V could be reached without exceeding 2-3 watts. This voltage was then maintained throughout the remaining time. Electrofocusing was continued until a low, constant current was reached. This usually meant a total running time of about one week.

The column was drained using a Harvard piston pump and collecting 3.0 ml fractions. The pH of each fraction was determined (also at 20°) and the enzyme activity located. The position of the enzyme activity with respect to the pH profile was used to establish the isoelectric point.

IV. RESULTS

A. Purification

One of the primary objectives of this research was to improve upon previously reported purification attempts and obtain homogeneous NTP-AMP tpp in high yields. Numerous purification techniques were investigated. The following steps, in the order given, were found to be the most successful.

1. Successful Purification Scheme

a) Extraction of the Enzyme from Mitochondria

NTP-AMP tpp was originally extracted from prepared mitochondria using the procedure described by Albrecht (17). This involved extraction of the mitochondria with PO_4^{-3} buffer to remove outer membrane enzymes, followed by sonication. With this method, however, we appeared to be losing a significant amount of NTP-AMP tpp activity in the discarded PO_4^{-3} extract. This led us to perform experiments relating to the intramitochondrial location of the enzyme, which in turn resulted in the formulation of a much improved extraction procedure. It consisted of treatment of the mitochondria with digitonin followed by extraction overnight with 0.075 M PO_4^{-3} and finally sonication. The combination of these three extracts produced initial yields of NTP-AMP tpp over three times those previously obtained.

b) Ammonium Sulfate Gradient Solubilization

The first step in the purification scheme, gradient solubilization of an ammonium sulfate-Celite column, was very successful in removing a

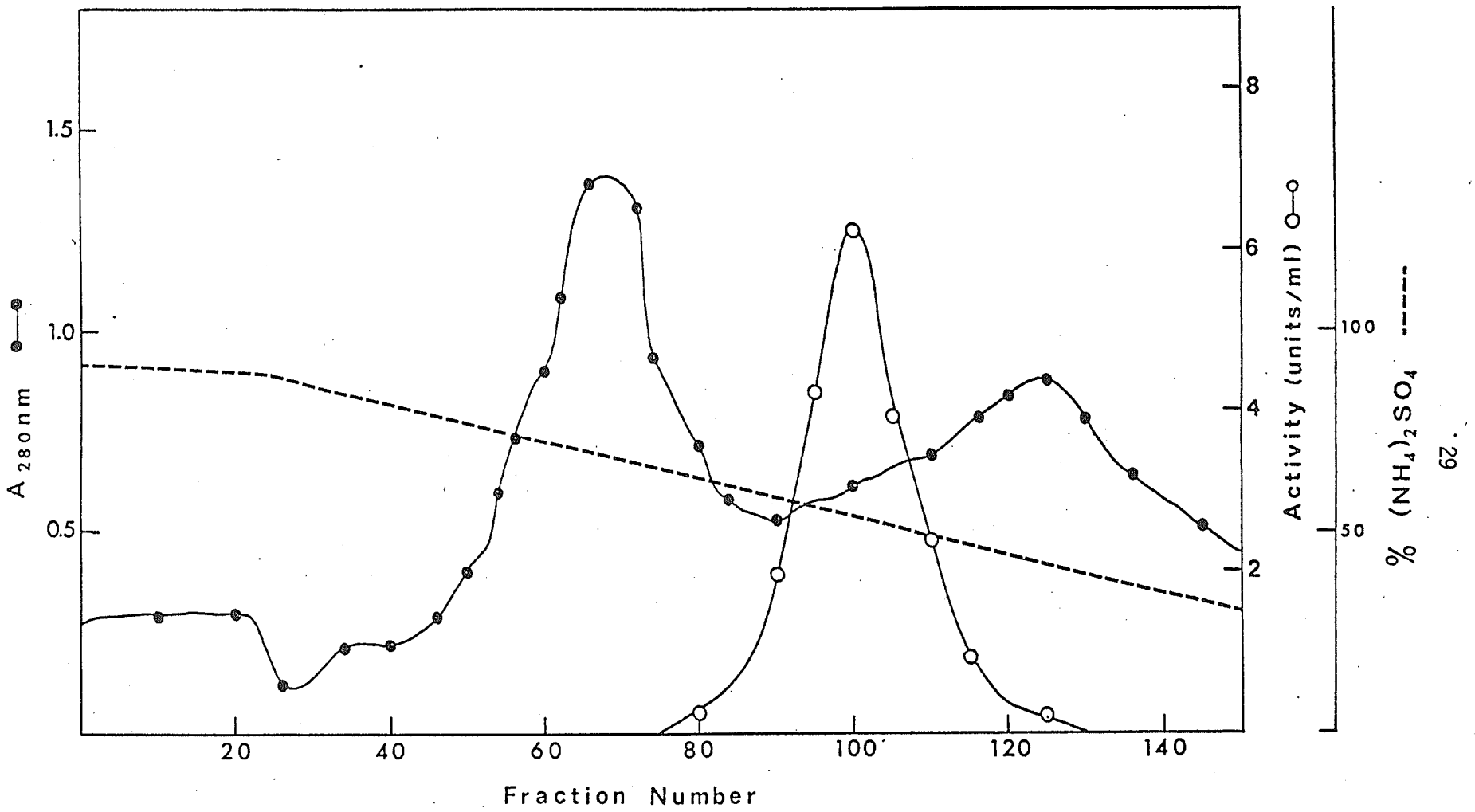


Figure 2. Ammonium sulfate gradient solubilization of NTP-AMP tpp on Celite support material. The chromatography was performed on a 2.5 x 100 cm. column, eluted at a flow rate of 65 ml/hr., and collecting 20 ml fractions. For further details see "Methods"

Note: Unless otherwise specified the enzyme referred to in the figures is inner and outer membrane enzyme.

large amount of contaminating proteins and reducing the volume of enzyme solution to a more easily handled quantity. Figure 2 shows a typical elution pattern obtained from such a chromatography. NTP-AMP tpp eluted between two large peaks of impurity protein, the removal of which usually resulted in a decrease in total protein of over 80%. This step is also beneficial in separating the enzyme from adenylate kinase, an enzyme that to some extent mimics NTP-AMP tpp.

It has been observed that NTP-AMP tpp (or at least some of it) is stabilized by the presence of ions such as $(NH_4)_2SO_4$. As the enzyme was usually solubilized in this chromatography between 55 and 40% $(NH_4)_2SO_4$ it was exposed to quite favourable conditions and recoveries of approximately 80% were common.

c) Sephadex G-75 Chromatography

The next step, gel filtration on Sephadex G-75, was found to be another very effective purification procedure. As shown in Figure 3 NTP-AMP tpp in such a chromatography eluted well behind an extremely large peak of higher molecular weight contaminating protein. After this step the total protein in the enzyme sample again was considerably reduced (often by greater than 90%) while the recovery of activity was good. This then resulted in a very large degree of purification.

The Amicon ultrafiltration steps following this proved to be a rapid and effective means of concentrating the enzyme. Provided slow stirring and a moderate nitrogen pressure of 20 psi were employed, less than 20% of the NTP-AMP tpp activity was usually lost and the concentration often even resulted in a slight purification of the enzyme.

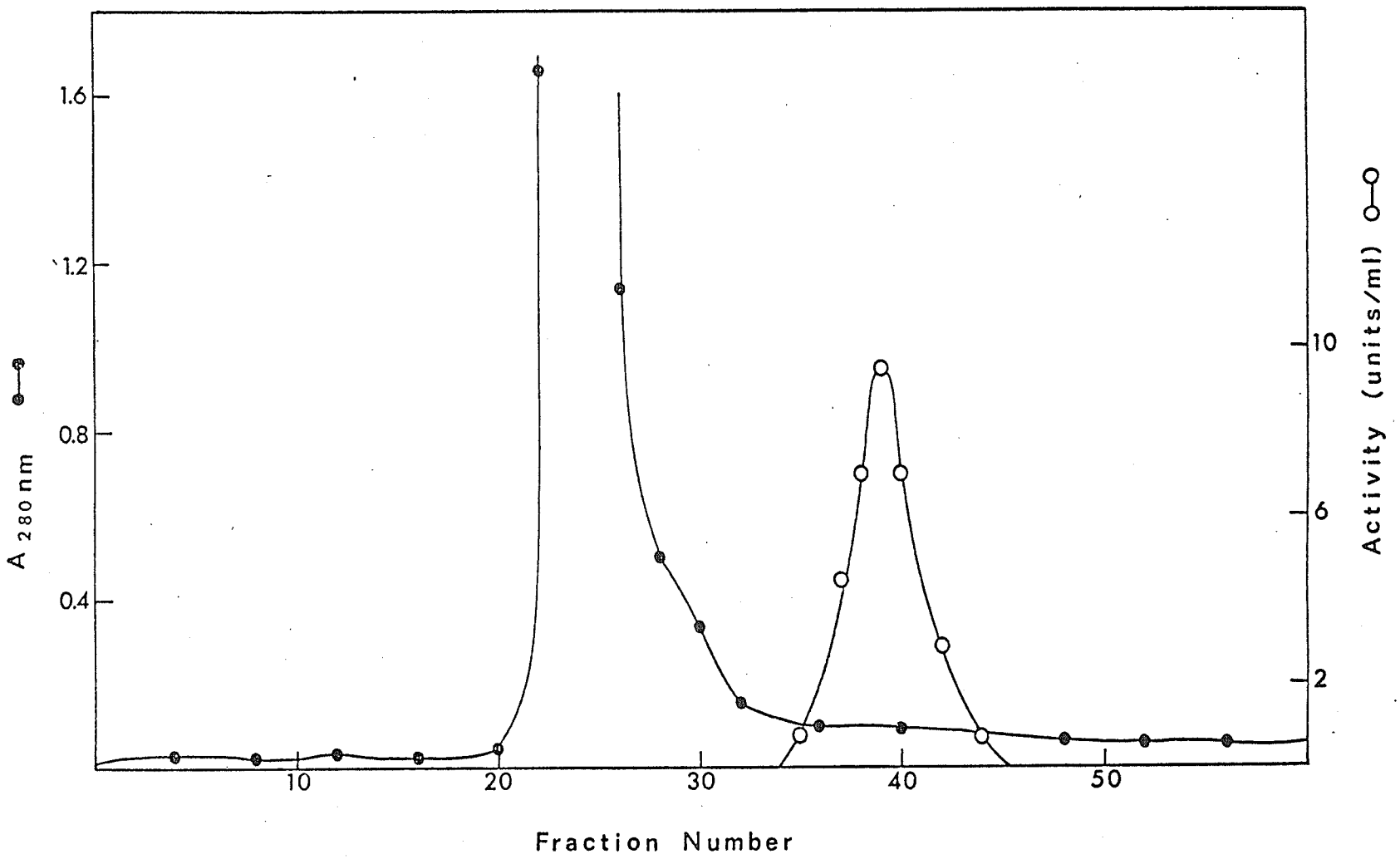


Figure 3. Purification of NTP-AMP tpp by Sephadex G-75 gel filtration. The chromatography was performed on a 5.0 x 100 cm. column, eluted at a flow rate of 55 ml/hr., and collecting 20 ml fractions.

d) Heat Treatment

The heat denaturation step used next took advantage of the enzyme's unusual heat stability. Even after 3 min. of heating at 65°, recoveries of NTP-AMP tpp of greater than 80% were regularly obtained. This procedure usually produced a 2 to 3-fold purification. Contrary to Albrecht's claim, the presence of substrate, AMP, in our case did not appear to further stabilize the enzyme.

e) SP-Sephadex Chromatography

Up to this point the purification steps had taken full advantage of differences in either solubility or molecular weight between impurity proteins and NTP-AMP tpp. Obviously what was also required to effectively purify the enzyme was a technique(s) separating proteins on the basis of charge differences. Finding such a technique proved to be a major difficulty. Chromatography on sulfopropyl-Sephadex, a strong cation exchanger, proved to be the only reasonably successful step of this sort we could find.

Even with such a strong ion-exchanger the enzyme did not bind under conditions at which it would be stable--0.1 M buffer, pH 7.0 (the enzyme's isoelectric point was found to be about 8.8 at 20°). Binding was achieved only by drastically reducing the pH to 6.0. In efforts to expose the enzyme to these conditions for as short a time possible, normal washing of the column with the binding buffer after sample application was eliminated. Instead, elution with a linear gradient of increasing NaCl concentration was begun immediately. Even so, recoveries from this step were usually poor--about 50%. The ratio of enzyme bound to the amount of ion-exchanger was very low and consequently the NTP-AMP tpp was

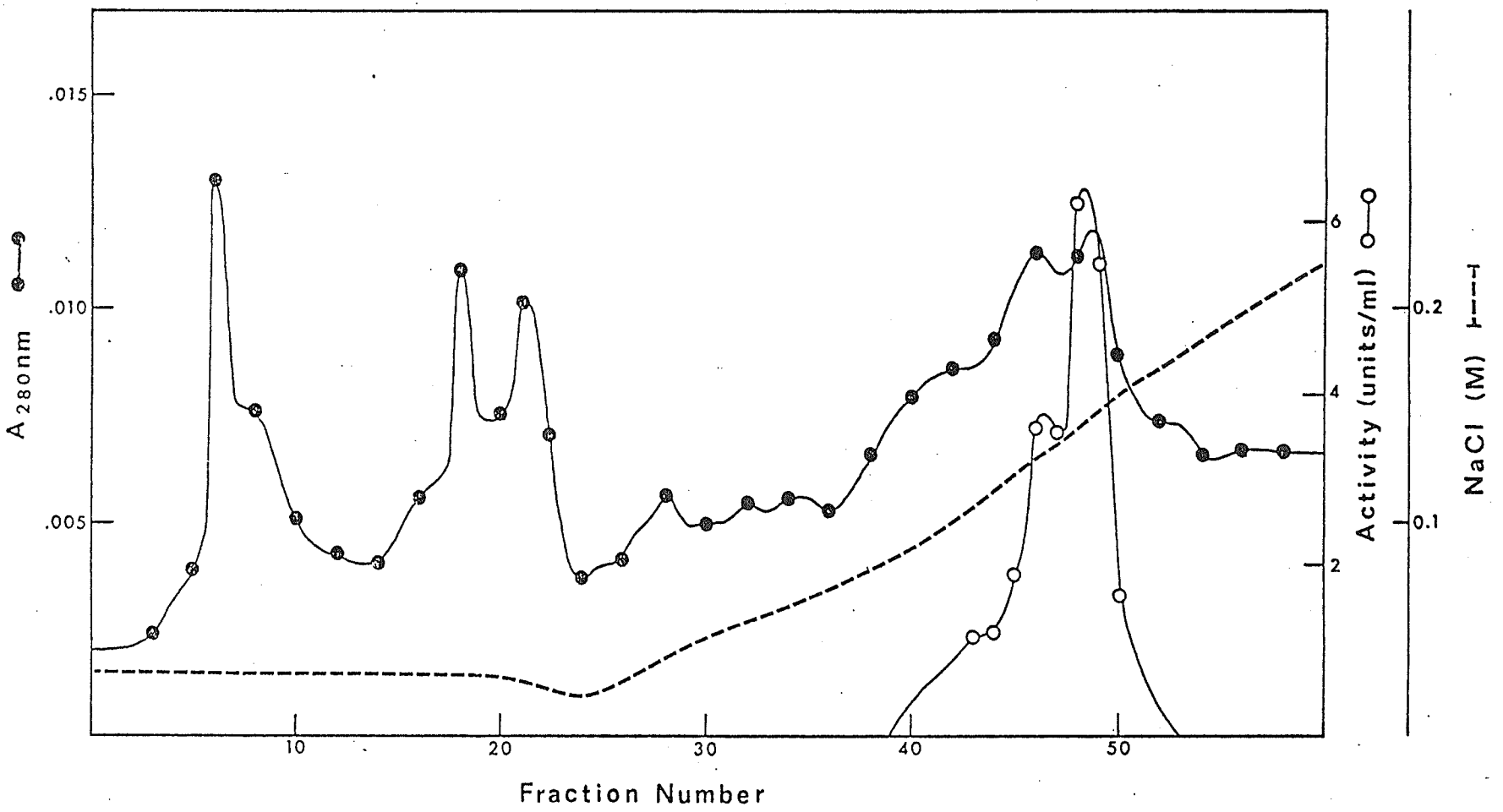


Figure 4. Purification of NTP-AMP tpp by SP-Sephadex chromatography. For this chromatography a 2.5 x 100 cm. column and a flow rate of 20 ml/hr. until one column bed volume had eluted and then 30 ml/hr. were used. 20 ml fractions were collected.

diluted considerably on elution. This in combination with the low pH likely produced the loss in activity observed.

A typical elution pattern for this type of chromatography is shown in Figure 4. The appearance of NTP-AMP tpp as two overlapping peaks will be discussed in the section dealing with the possibility of two forms of NTP-AMP tpp, p. 61. At least two peaks of foreign protein usually eluted before NTP-AMP tpp. The first peak appeared before a bed volume of buffer had passed through the column and probably corresponded to anionic species. A second peak of unbound protein would appear as one bed volume had been eluted. Despite the poor yields this chromatography was successful in purifying the enzyme from at least some proteins of different charge properties.

f) Sephadex G-75 Superfine Chromatography

The final chromatography on the more resolving Sephadex G-75, superfine, was used to separate from NTP-AMP tpp any remaining major impurities. In this case (see Figure 5) NTP-AMP tpp eluted in high yields and well separated from several peaks of higher molecular weight. The protein and enzyme activity profiles of the NTP-AMP tpp peak were coincident and very symmetrical. Also the specific activity was found to be approximately constant throughout the peak. This suggested that the enzyme was very close to homogeneity.

g) Summary of the Purification

Table 1 summarizes the percent recoveries, degree of purification, and other data obtained after each step in a typical purification of NTP-AMP tpp. The Celite and first Sephadex G-75 chromatographies stand out as the two most effective purification steps, producing respectively 5- and 17-fold purifications. In the end this scheme results in enzyme over 400-fold purified and with a specific activity of 485 units/mg. Unfortunately, even

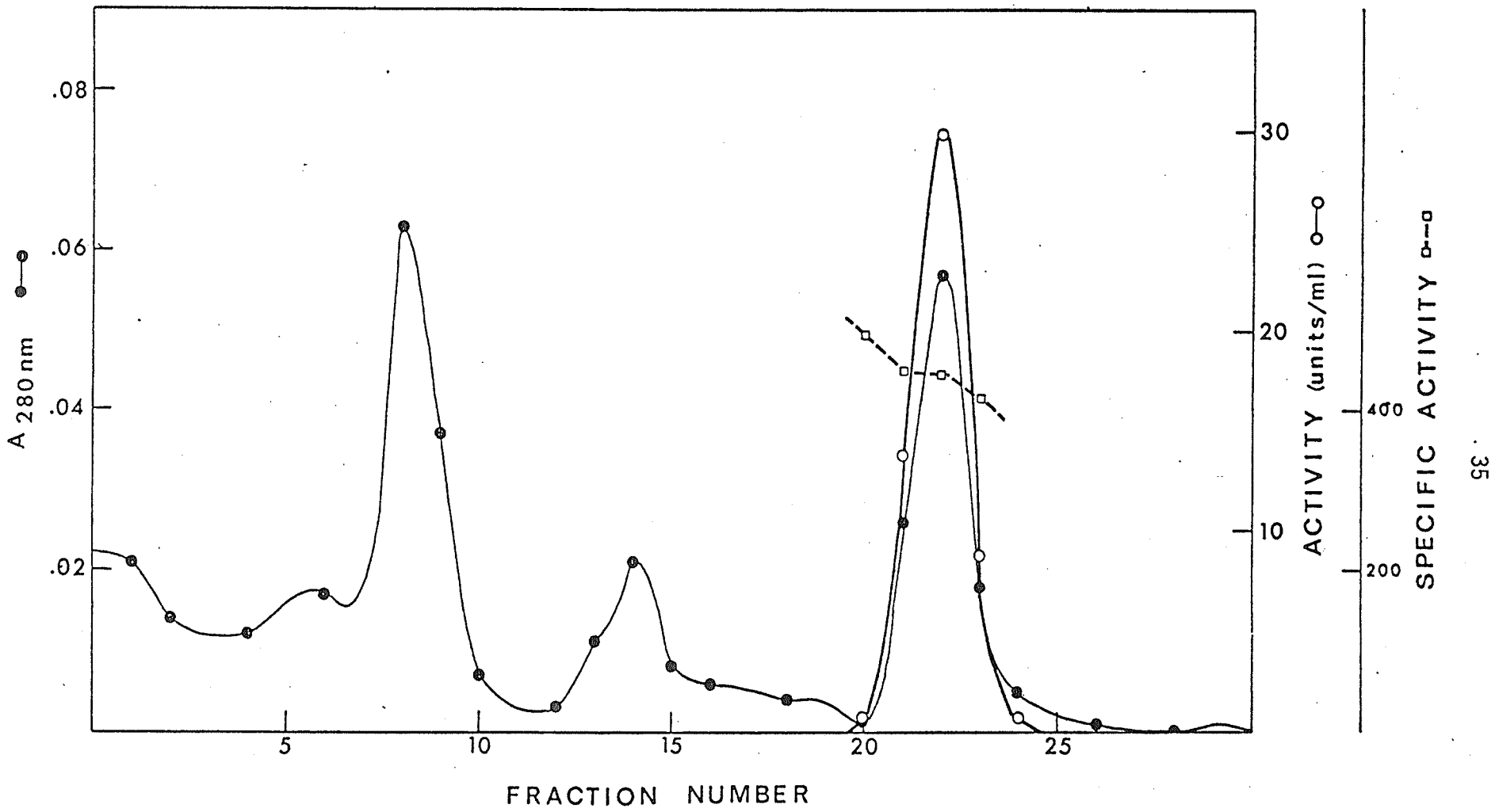


Figure 5. Purification of NTP-AMP tpp by Sephadex G-75 superfine chromatography. A 2.5 x 100 cm. column was eluted at a flow rate of 26 ml/hr., collecting 10 ml fractions.

TABLE 1

Summary of the Purification of NTP-AMP tpp

Step	Volume (ml)	Total Units	Total Protein (mg)	Specific Activity (units/mg)	% Recovery	fold-Purification
Combined Extracts	1785	3340	2934	1.14	100	1
Celite	620	2695	474	5.69	81	5.0
Sephadex G-75	220	2202	22.9	96	66	84
Concentration	22	1751	11.3	155	52	136
Heat Treatment	5.4	1497	4.58	327	45	287
SP-Sephadex	2.0	606	1.52	399	18	350
Sephadex G-75 super-fine	0.8	461	0.95	485	14	425

with a reasonable overall recovery of 14% less than one mg of enzyme is produced from two kg of beef heart.

h) Analytical Procedures

SDS gel electrophoresis and gel isoelectric focusing were used to monitor the progress of the purification. Figure 6 shows the typical SDS patterns obtained after each step and illustrates the number of contaminating proteins with which we had to contend.

i) Homogeneity

The appearance of the NTP-AMP tpp peak after Sephadex G-75 superfine chromatography and the almost constant specific activity suggested that the final enzyme produced was close to, if not, homogeneous. In order to further assess the homogeniety of the prepared enzyme it was subjected to three systems of gel electrophoresis: SDS gel electrophoresis, gel isoelectric focusing, and pH 4.3 cathodic disc gel electrophoresis. The results are shown in Figure 7. A single band was obtained in SDS gel electrophoresis. In gel isoelectric focusing one major band and two very small bands of lower isoelectric point appeared. One dark band and two fainter ones of higher mobility were also obtained in cathodic disc gel electrophoresis. It appears that our NTP-AMP tpp preparation contains two minor impurities but is very nearly homogeneous (we estimate it to be 95-99% pure).

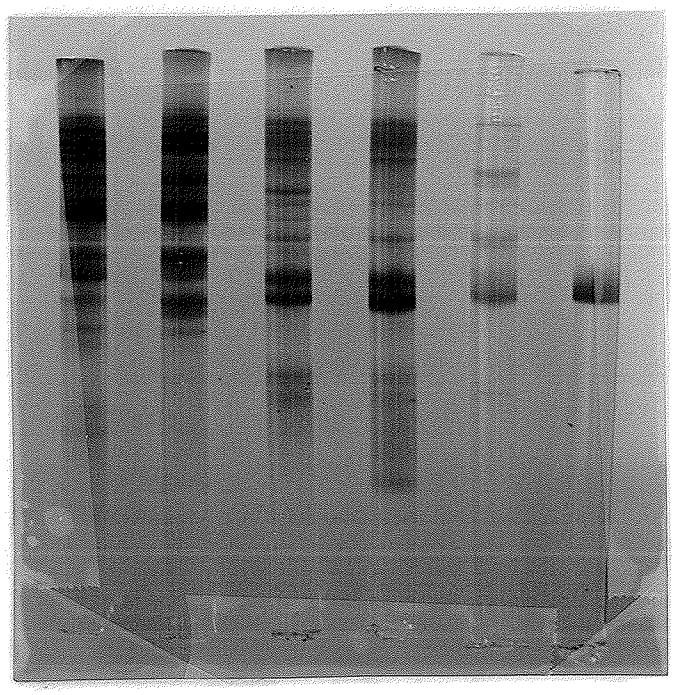


Figure 6. SDS gel electrophoresis after each step in the purification. 1. combined extracts 2. after ammonium sulfate gradient solubilization on Celite 3. after Sephadex G-75 chromatography 4. after heat treatment 5. after SP-Sephadex 6. after Sephadex G-75 superfine. Sample loads used were 100, 40, 20, 20, 20, and 30 µg, respectively.

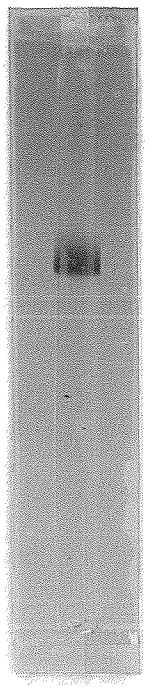


Figure 7a. SDS gel electrophoresis of purified NTP-AMP tpp.

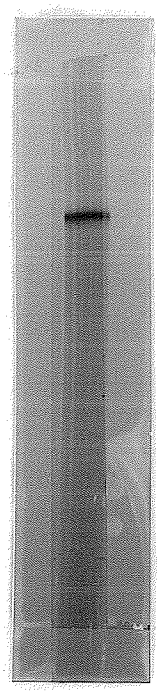


Figure 7b. Gel isoelectric focusing of purified NTP-AMP tpp.

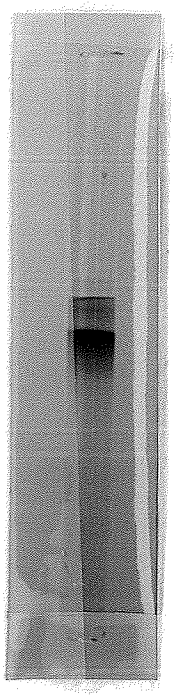


Figure 7c. pH 4.3 cathodic disc gel electrophoresis of purified NTP-AMP tpp.

2. Unsuccessful Purification Techniques

Numerous other purification techniques were also tested but they proved unworkable and were therefore abandoned. Table 2 gives a complete list of the techniques and conditions tried and their results. From some of these attempts we were at least able to gain insight into characteristics of the enzyme.

In cases where low ionic strengths were used to try to bind the enzyme to either a cation exchanger such as phosphocellulose or an adsorbent such as hydroxylapatite, drastic losses in activity were noted. This suggested an instability of NTP-AMP tpp in the presence of low ionic strengths which we subsequently investigated. The main conclusion of this experiment was just what was expected--that NTP-AMP tpp loses activity rapidly under conditions of low ionic strength.

This meant that the commonly used approach for ion-exchange or adsorption chromatography--that is, binding the protein at low ionic strength and then desorption by increasing the ionic strength or changing the pH of the elution buffer--would not be suitable for NTP-AMP tpp. Two alternate procedures were therefore tried.

The first was to attempt negative binding by using conditions favourable to NTP-AMP tpp but under which impurity proteins and not NTP-AMP tpp would bind to the exchanger or adsorbent. Unfortunately, under such conditions even the impurities could not be induced to bind.

The second alternative involved trying to bind the enzyme to a cation exchanger by using a buffer of moderate ionic strength but with a pH lower than the enzyme's isoelectric point of 8.8. This failed with CM-Sephadex but was finally fairly successful with the strong cation

TABLE 2

Unsuccessful Purification Attempts

Technique	Conditions Used	Reference	Results
1. Ion-exchange	Binding with:		
(1) DEAE-cellulose negative binding	0.005 M TEA-Cl, pH 7.5	17.	very poor recoveries of NTP-AMP tpp
(2) Phosphocellulose positive binding	0.001 M PO_4^{-3}, pH 7.5		very poor recoveries
(3) CM-Sephadex negative or positive binding	0.1 M Tris-PO_4, pH 7.5		no binding of NTP-AMP tpp or other protein
(4) SP-Sephadex positive binding	0.1 M Tris-PO_4 pH 7.5		negligible binding
	0.1 M Tris-PO_4 pH 7.0		a little binding
2. Adsorption	Binding with:		
(1) Hydroxylapatite positive binding	0.01 M PO_4^{-3}, pH 7.5		some binding but poor recovery of NTP-AMP tpp
negative	0.09 M PO_4^{-3}, pH 7.5		no binding
(2) Alumina C	0.1 M Tris-Cl, pH 7.5	89.	NTP-AMP tpp bound well but could not be completely desorbed
3. Sephadex G-50 (medium and superfine) and G-100	0.1 M Tris-Cl pH 7.5		no separation from impurity proteins
4. Ion Filtration (combination of ion-exchange and gel filtration)	0.01 M PO_4^{-3}- 0.25 M KCl pH 7.5	90.	retarded on column but not separated from other impurities
5. Crystallization		91.	no crystallization but activity at 53% $(NH_4)_2SO_4$

exchanger, SP-Sephadex. The pH had to be reduced to 6.0 and even then the capacity of the exchanger for the enzyme was very low. This seems to indicate the absence of a large net positive charge on the enzyme even several pH units away from its isoelectric point.

Attempts were also made to crystallize the enzyme using the technique of Jakoby (91) in which the protein is completely precipitated with $(NH_4)_2SO_4$ and then extracted with solutions of decreasing $(NH_4)_2SO_4$ saturation. The extracts are then observed for crystal growth. Although no crystallization was obtained, most of the NTP-AMP tpp activity was found at 53% $(NH_4)_2SO_4$ indicating this to be the $(NH_4)_2SO_4$ concentration of minimum solubility. That it did not crystallize was likely due to the impurity of the sample used. It seems likely, however, that if enough purified enzyme were used crystals of NTP-AMP tpp could be readily obtained by this technique.

B. Characterization

While attempting to purify NTP-AMP tpp, a number of its properties were also investigated. These included general stability properties necessary for the proper handling of the enzyme, properties relating to its location and concentration in mitochondria, and some of its physical characteristics.

1. pH Stability

In order to determine the optimum pH at which to maintain NTP-AMP tpp, its stability at various pH's was investigated. Equal amounts of enzyme were incubated in buffers of various pH's for 30 min. and then assayed. This was done at both 5° and 30°. Figure 8 shows the plots of relative activity versus pH obtained for the two temperatures.

The pH of optimum stability was found to depend on the temperature. At 30° the enzyme showed a broad range of stability, from pH 5 to 11, with an optimum at pH 7.5. At 5°, however, the enzyme had a much narrower stability range and its optimum was shifted to a higher pH, 8.5.

2. Temperature Stability

NTP-AMP tpp was found to be generally unstable at cold-room temperatures (0-5°). This was reflected in its narrower pH stability range at 5°. Also previously frozen mitochondria failed to yield any active enzyme.

On the other hand, the enzyme was seen to be extremely stable at room temperature and higher temperatures. All other conditions being optimum, extremely good yields of enzyme were obtained from chromatographies performed at room temperature. In heat denaturation steps NTP-AMP tpp

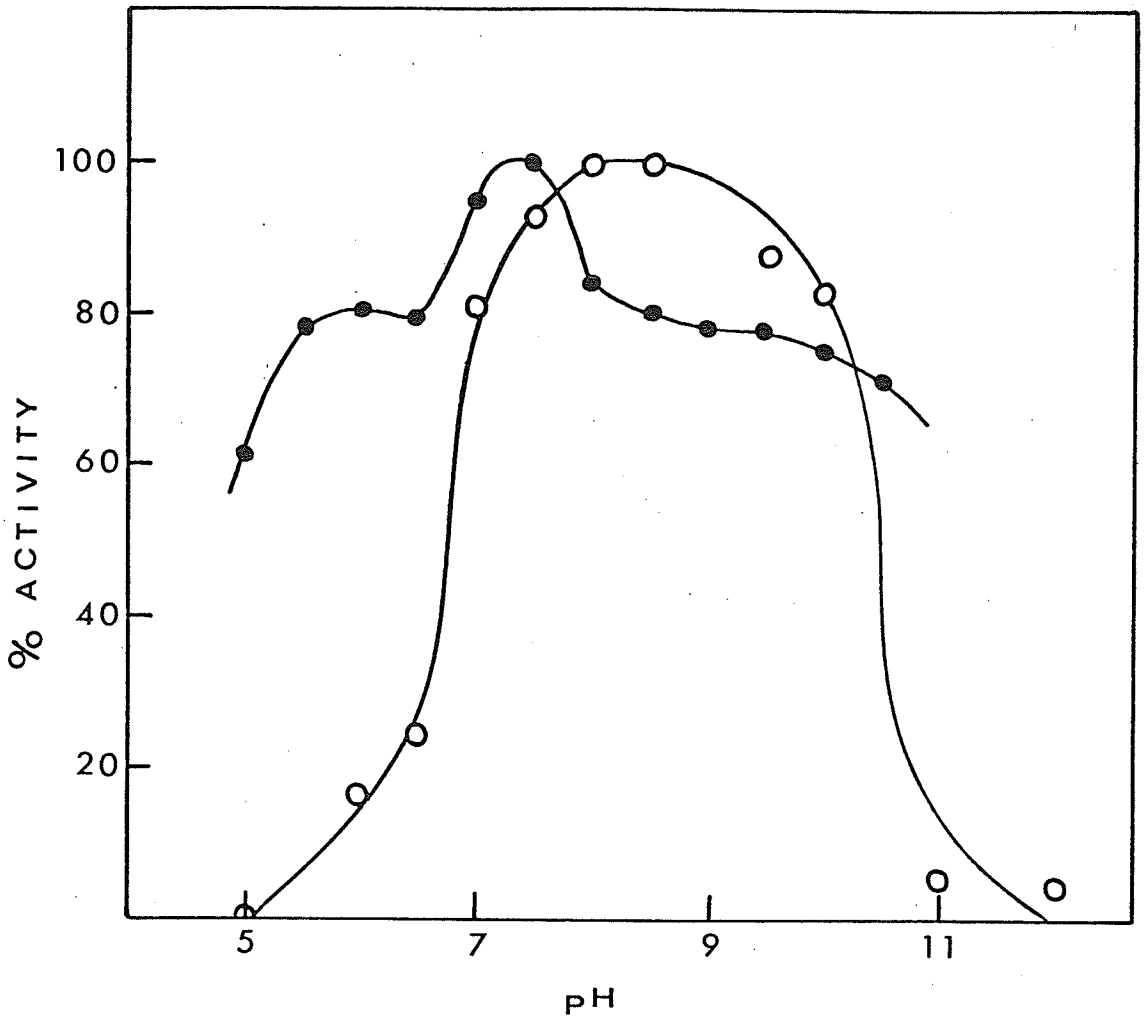


Figure 8. pH stability curves for NTP-AMP tpp at 5° and 30°. Only inner membrane enzyme was used here. Succinate buffers were used for the pH range 5.0-6.0, imidazole for pH 6.5-7.0, Tris for pH 7.5-8.5, glycine for pH 9.0-10.5, and sodium phosphate for pH 11.0-12.0. The highest activity obtained was taken as 100%. All buffers were 0.1 M. ● corresponds to 30° and o to 5°.

was heated for 3 min. at 65° and still regularly remained 80-100% active.

3. Ionic Strength Stability

The results of certain purification attempts had suggested that NTP-AMP tpp might be unstable at low ionic strengths. To test this a systematic study of the enzyme's stability at various ionic strengths was made. It involved chromatographing enzyme samples on small Sephadex G-25 columns equilibrated with Tris-PO_4 buffers (pH 7.5) of various molarities. This served both to desalt the enzyme and expose it to the incubating buffer. The activity of the enzyme was then assayed at several time intervals after elution.

Figure 9 shows plots of the percent activity remaining versus time for three buffers. NTP-AMP tpp appears to be quite stable at the higher ionic strengths, 0.05 and 0.1 M Tris-PO_4; its activity decreased only moderately under these conditions. However, at 0.01 M Tris-PO_4 its activity decreased rapidly with time. This shows conclusively that NTP-AMP tpp is unstable when exposed to conditions of low ionic strength.

4. Intramitochondrial Location

Lima and Vignais (16) had shown NTP-AMP tpp to be located in both the outer and inner membrane compartments of rat liver mitochondria. Albrecht (17), however, concluded that it was entirely bound to the inner membrane of bovine heart mitochondria. In our initial procedure for extraction of mitochondria we followed the method outlined by Albrecht in which the mitochondria were pre-extracted with PO_4^{-3} buffer to remove outer membrane enzymes and then sonicated to release the NTP-AMP tpp.

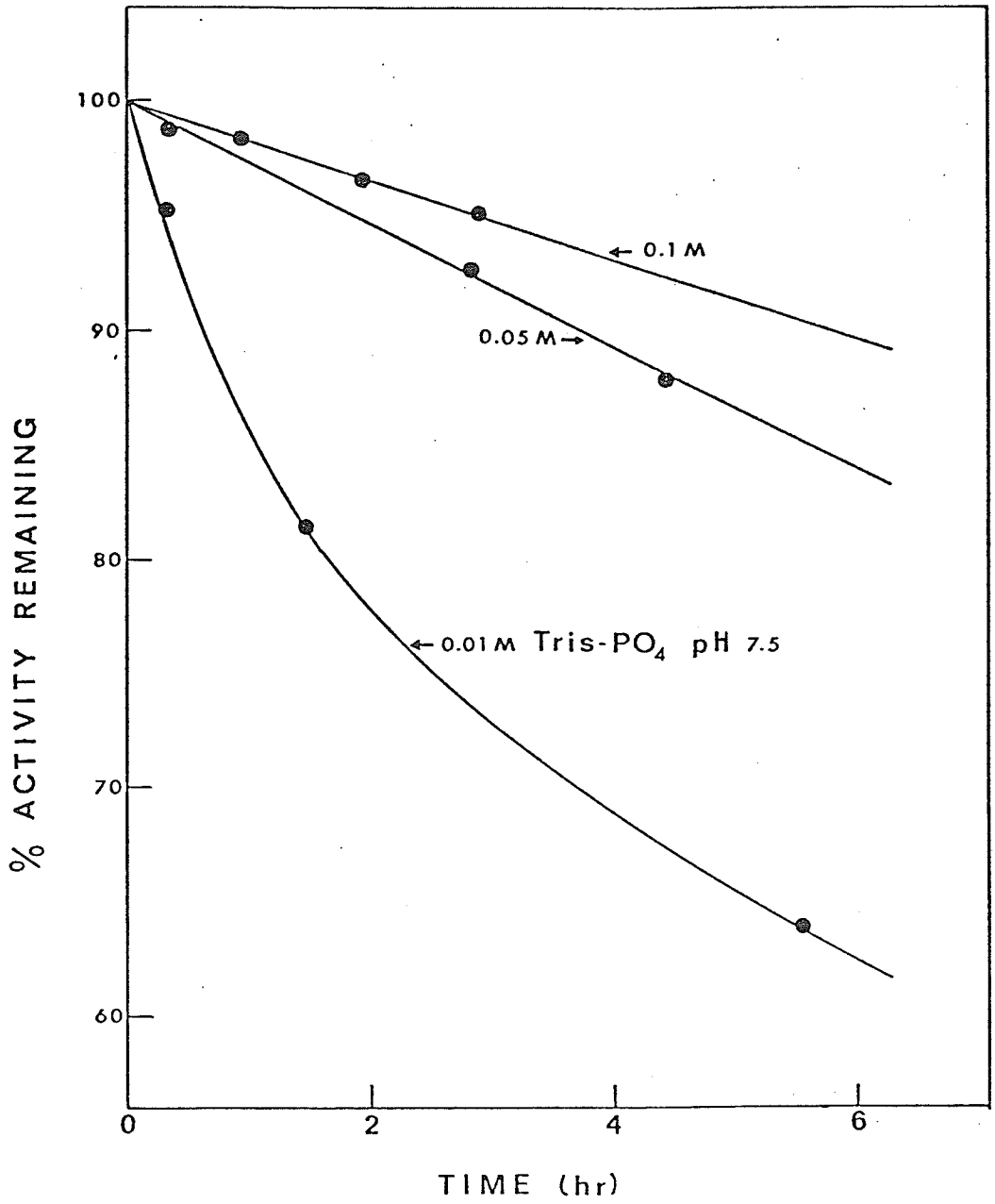


Figure 9. The stability of NTP-AMP tpp at various ionic strengths. 100% activity corresponds to the activity of the enzyme just as it was applied to the Sephadex G-25 column.

We noticed, however, that a significant amount of NTP-AMP tpp activity was being discarded in the PO_4^{-3} extract. This suggested to us that in analogy to Lima's findings, a proportion of NTP-AMP tpp was also located in the bovine heart mitochondrial outer membrane region. In hopes of not only clearing up the ambiguity but also of obtaining a more effective means of extracting the enzyme it was decided to investigate more thoroughly the question of intramitochondrial location.

An investigation was first made of the extraction of the enzyme by PO_4^{-3} buffer. The amount of enzyme released by buffers of differing PO_4^{-3} concentrations was compared. A plot of this is shown in Figure 10. The enzyme extracted was found to increase with PO_4^{-3} concentration, reaching a maximum at 0.075 M and then declining. The optimum concentration turned out to be precisely that recommended by Albrecht to remove outer membrane enzymes and is the concentration used since to perform any NTP-AMP tpp extractions.

To settle the location question the enzyme released on treatment of mitochondria with digitonin was measured. Digitonin is a detergent which reacts with cholesterol and because the cholesterol content of mitochondrial outer membranes is so much higher than that of inner membranes (92) at moderate concentrations digitonin ruptures and eventually removes preferentially the outer membranes.

It was found that treatment of mitochondria with digitonin did in fact result in the release of NTP-AMP tpp into the medium confirming its presence in the outer membrane compartment. Figures 11 and 12 show plots of the activity and specific activity of enzyme released at varying digitonin concentrations. The amount of enzyme released was found to be proportional

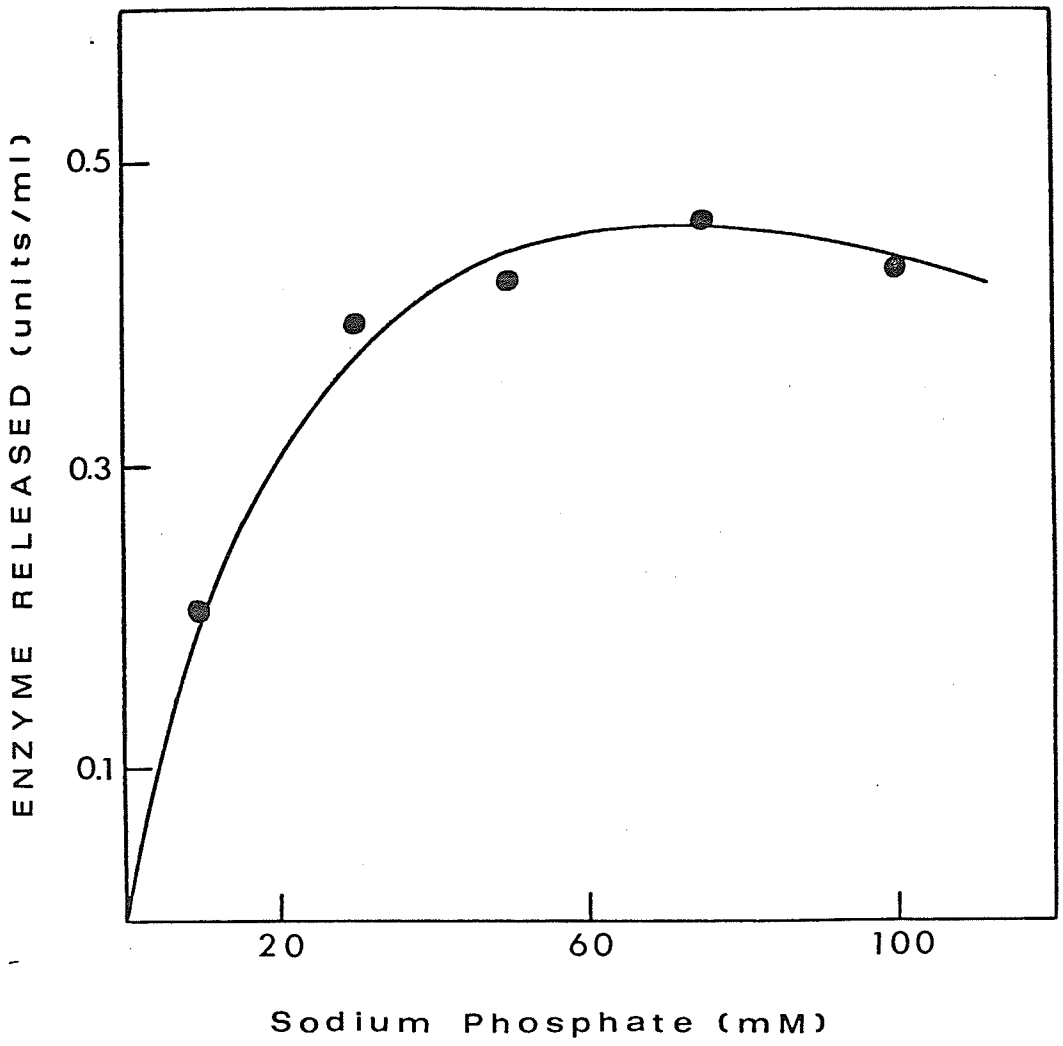


Figure 10. The NTP-AMP tpp activity released from mitochondria incubated in various concentrations of sodium phosphate buffer. Equal volumes of mitochondria were suspended in 9 volumes of phosphate buffers of different concentrations and incubated overnight. The phosphate extracts were obtained by centrifugation (26,000 x g for 20 min.) and assayed for NTP-AMP tpp activity.

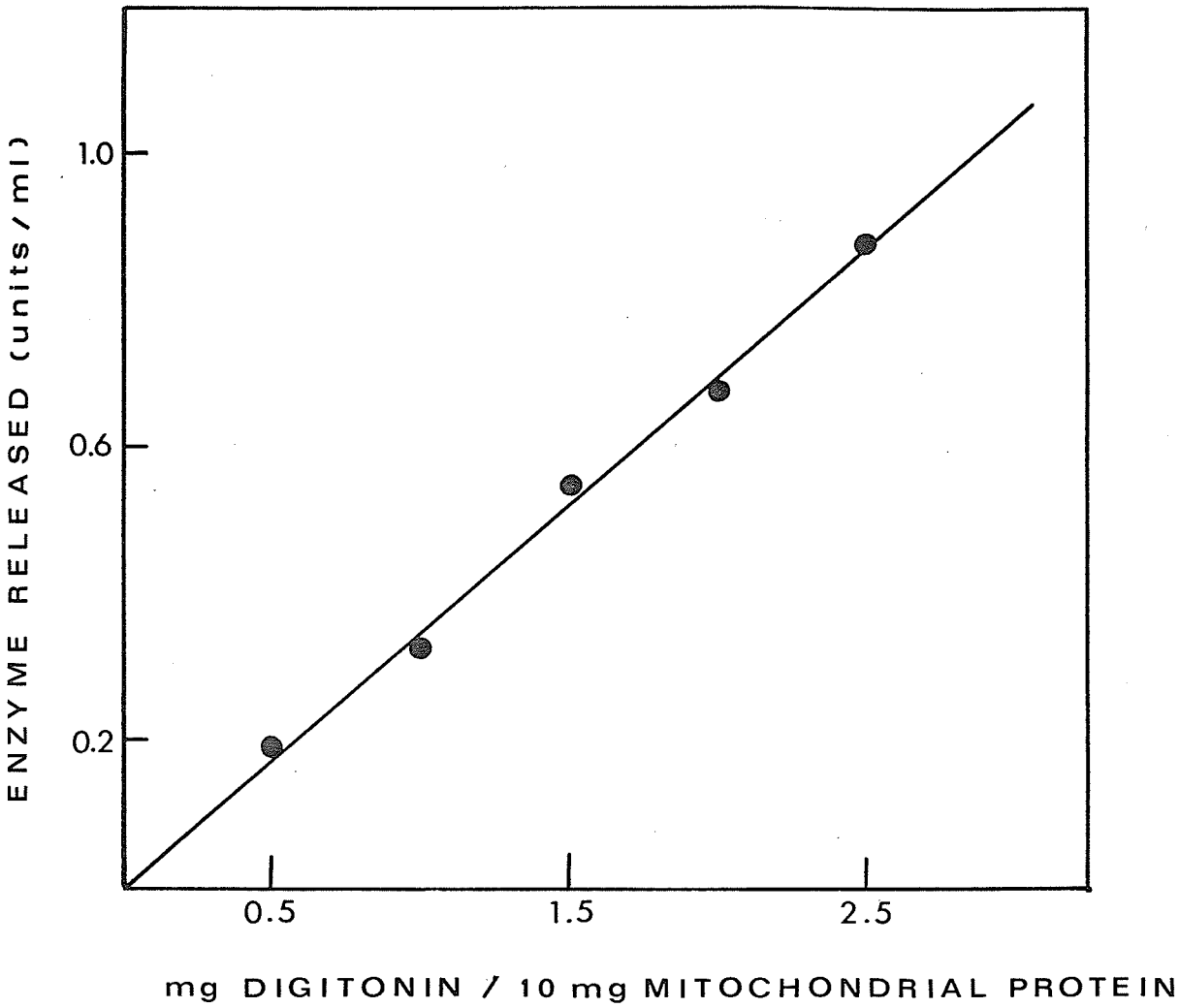


Figure 11. The NTP-AMP tpp activity released by treatment of mitochondria with various concentrations of digitonin. Mitochondria were treated with different concentrations of digitonin as described in the "Extraction" section of "Methods". The digitonin extracts were obtained by centrifugation and assayed for NTP-AMP tpp activity.

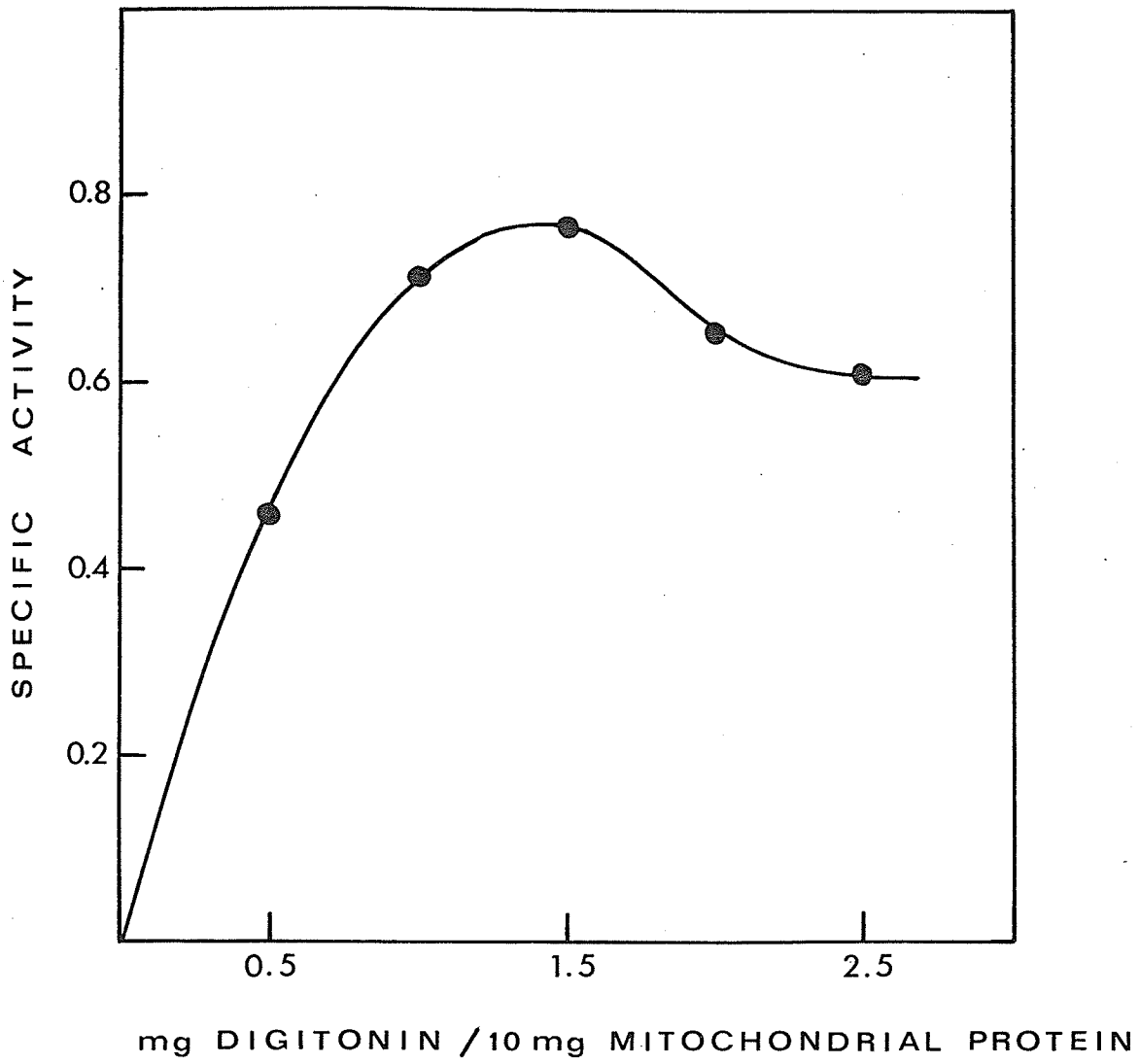


Figure 12. The specific activity of NTP-AMP tpp released by treatment of mitochondria with various concentrations of digitonin.

to digitonin concentration. The specific activity reached a maximum at 1.5 mg of digitonin per 10 mg of mitochondrial protein and then declined.

The effect of incubation time during the digitonin treatment on the amount of enzyme released was also investigated. Mitochondria were incubated with digitonin at a concentration of 1.5 mg/ 10 mg protein for 20, 40, and 60 min. The enzyme released was then compared. The enzyme activity released was found to increase with increasing incubation time until reaching a plateau at 40 min.

0.057 units of NTP-AMP tpp were released per mg of mitochondrial protein using a ratio of digitonin to mitochondrial protein of 1.5 mg to 10 mg and incubating for 40 min. An estimate of the total enzyme content of mitochondria was made by solubilization with a 0.02 M deoxycholate-10% KCl solution. Mitochondria were found to contain approximately 0.240 units of NTP-AMP tpp activity per mg of protein. This means that the digitonin released 24% of the NTP-AMP tpp or that at least 24% of NTP-AMP tpp is located in the outer membrane of mitochondria.

5. Total Content of the Enzyme in Mitochondria

In an attempt to determine the total concentration of NTP-AMP tpp in bovine heart mitochondria three techniques for solubilizing mitochondrial membranes were employed (93). These involved treatment of known amounts of mitochondrial protein with tert-amyl alcohol, Triton X-100, and deoxycholate-KCl solutions. The exact procedures used are described in the methods section. The table on the next page summarizes the units of NTP-AMP tpp per mg of mitochondrial protein released by each of these agents.

TABLE 3

NTP-AMP tpp Activity Released from Mitochondria by Solubilizing Agents

solubilizing agent	units/mg of protein
t-amyl alcohol	0.083
Triton X-100	0.200
Deoxycholate-KCl	0.240

Obviously the t-amyl alcohol and Triton X-100 treatments were either much less effective than deoxycholate-KCl in solubilizing the mitochondria or resulted in denaturation of the enzyme. The value obtained with treatment by deoxycholate-KCl was taken as an approximation of the total mitochondrial concentration of the enzyme.

6. Molecular Weight

Gel filtration and SDS gel electrophoresis were used to determine both the native and subunit molecular weights of NTP-AMP tpp.

a) Gel Filtration

Gel filtration studies were performed using Sephadex G-75 and the following different buffer systems:

(i) low ionic strength--0.01 M potassium phosphate, pH 7.0

(ii)high ionic strength--0.01 M potassium phosphate and 0.2 M KCl, pH 7.0

(iii) Albrecht's conditions--0.2 M potassium phosphate pH 7.5

For each system marker proteins and NTP-AMP tpp were chromatographed and calibration curves of log (molecular weight) versus K_{av}, their partition coefficients, obtained. The markers and their corresponding molecular weights were: bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen A, 25,700; and ribonuclease A, 13,700. The molecular

weights used were those cited by Weber and Osborn (76).

Figure 13 shows a typical elution pattern obtained for one of the buffer systems. All peaks were symmetrical and quite well separated. Also the marker proteins chosen spanned well the range of molecular weights around that of NTP-AMP tpp. This can also be seen in the calibration curve shown in Figure 14.

The table below gives the molecular weights of NTP-AMP tpp determined in each system.

TABLE 4

Molecular Weights of NTP-AMP tpp Determined by Gel Filtration

System	Molecular Weight
i) low ionic strength	28,500 (2)*
ii) high ionic strength	29,800 (2)
iii) Albrecht's conditions	30,000 (1)

* indicates the number of determinations

All the molecular weights found were close to 30,000. That the molecular weight at high and low ionic strength was the same indicated that no ionic strength dependent association or dissociation of the protein was occurring.

Albrecht (17) had reported a molecular weight for NTP-AMP tpp of 52,000, determined by Sephadex G-200 gel filtration. Because his value was considerably higher than those we were finding, we repeated our molecular weight determination under the same buffer conditions he had used. A molecular weight of 30,000 was again obtained.

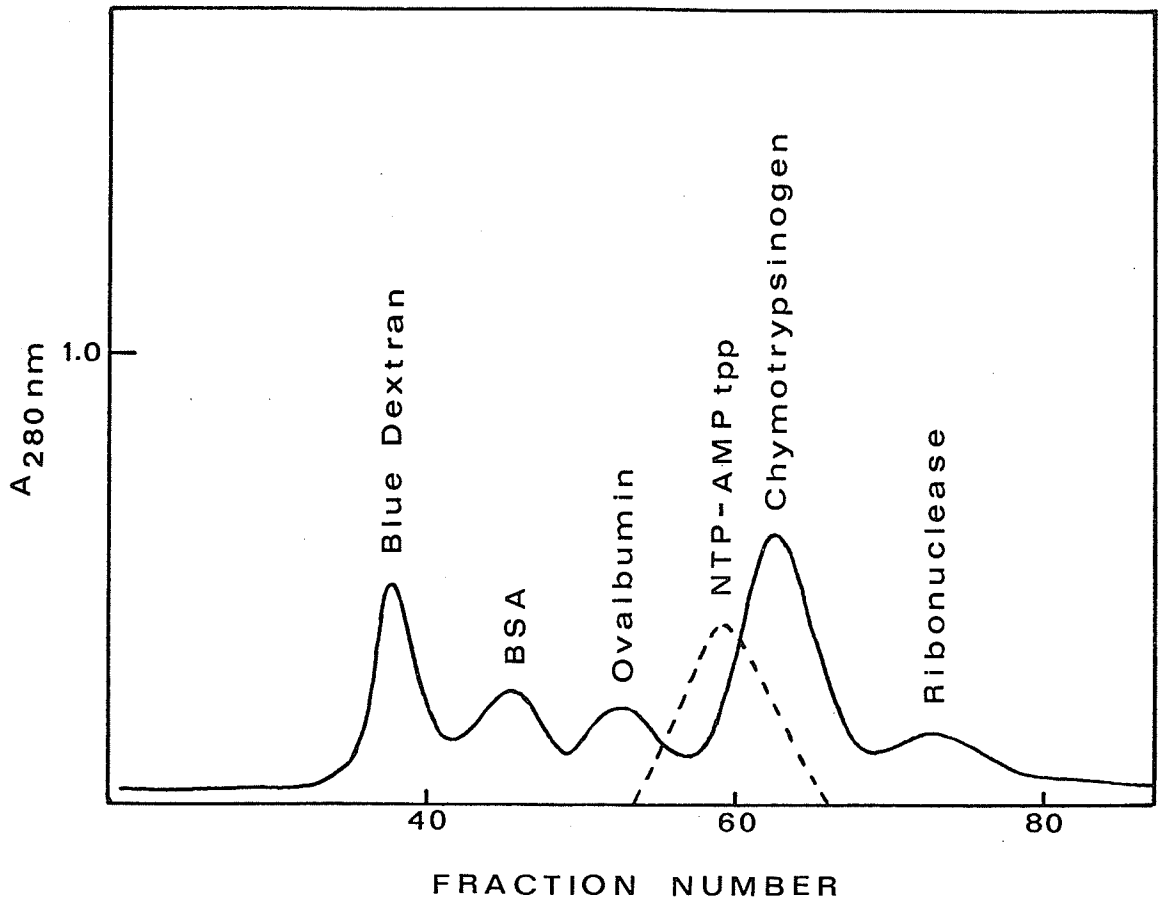


Figure 13. A typical elution pattern obtained for the molecular weight determination of NTP-AMP tpp by Sephadex G-75 gel filtration. The pattern represents a composite of several chromatographies on a 2.5 x 100 cm. column of Sephadex G-75 in the high ionic strength buffer system. The absorbance at 280 nm was obtained from continuous monitoring by an absorbance monitor.

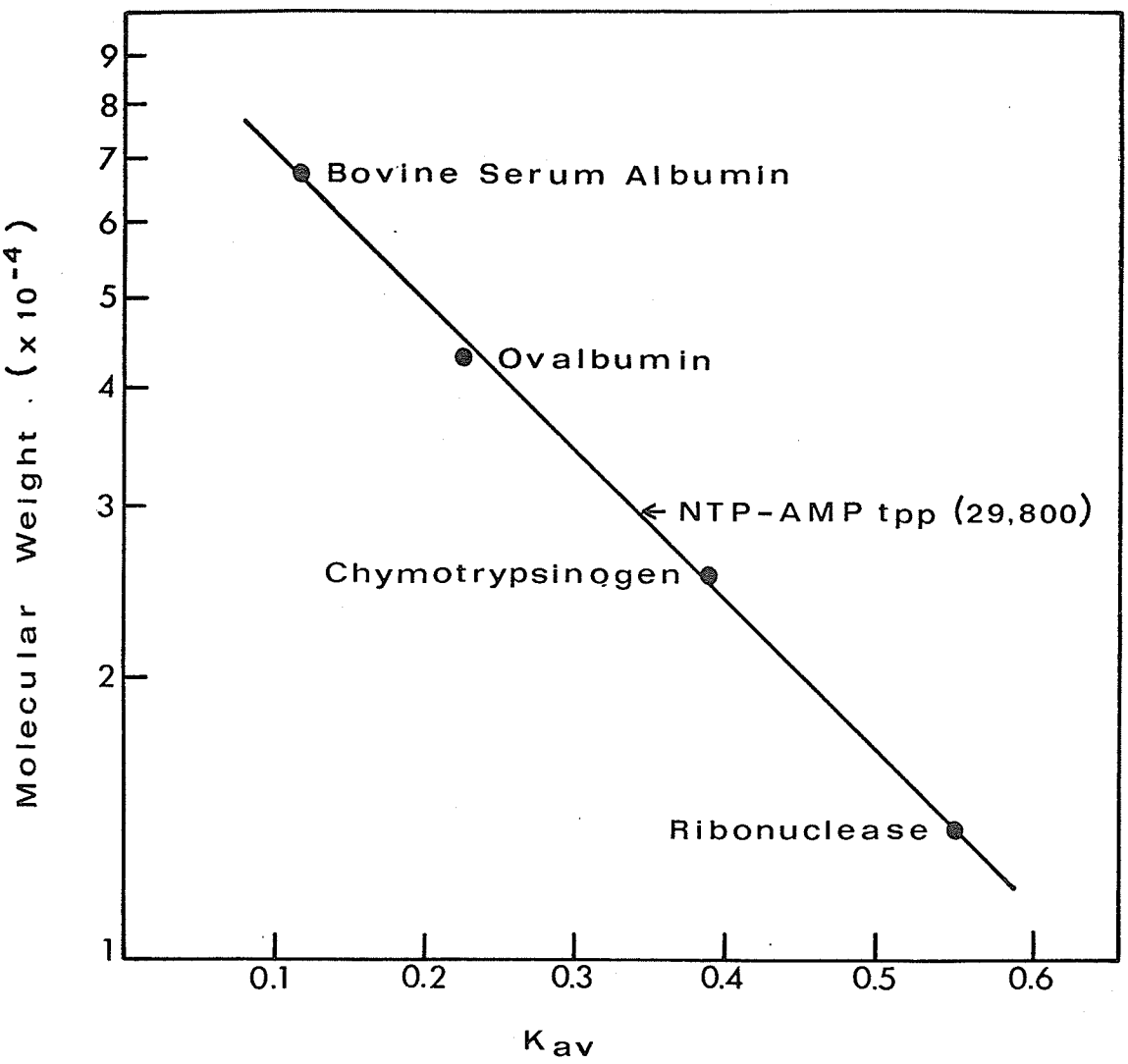


Figure 14. A calibration plot for the molecular weight determination of NTP-AMP tpp by Sephadex G-75 gel filtration. This was the plot obtained for the high ionic system. For the definition of K_{av} see "Methods".

b) SDS Gel Electrophoresis

When electrophoresed on standard Weber and Osborn type SDS polyacrylamide gels the purified NTP-AMP tpp appeared as only one band The following marker proteins were also run: rabbit muscle creatine kinase, M.W. 41,300; lactate dehydrogenase, M.W. 36,000; α-chymotrypsinogen A, M.W. 25,700; β lactoglobulin, M.W. 18,400; and myoglobin, M.W. 17,200. The molecular weight for the creatine kinase was taken from (94) while the other molecular weights were obtained from Weber and Osborn (76).

A calibration plot was made of log(molecular weight) versus the mobility of the markers as shown in Figure 15. From this a molecular weight of 28,000 was found for NTP-AMP tpp. This agreed very well with the gel filtration values. Also, that no bands of lower molecular weight appeared under these conditions demonstrated that NTP-AMP tpp exists as a monomer.

7. Isoelectric Point

The isoelectric point of NTP-AMP tpp was determined from isoelectric focusing in both a sucrose density gradient and polyacrylamide gels.

a) Gel Isoelectric Focusing

The isoelectric point of the enzyme was determined from gel electrofocusing by comparison to the following marker proteins of known isoelectric point: cytochrome c, 9.8-10.1; α-chymotrypsinogen A, 9.1; myoglobin, 6.99; and ovalbumin, 4.59. The isoelectric point of α-chymotrypsinogen A was found in (95) and the others' in (96). Figure 16 shows the calibration curve of mobility versus isoelectric point from

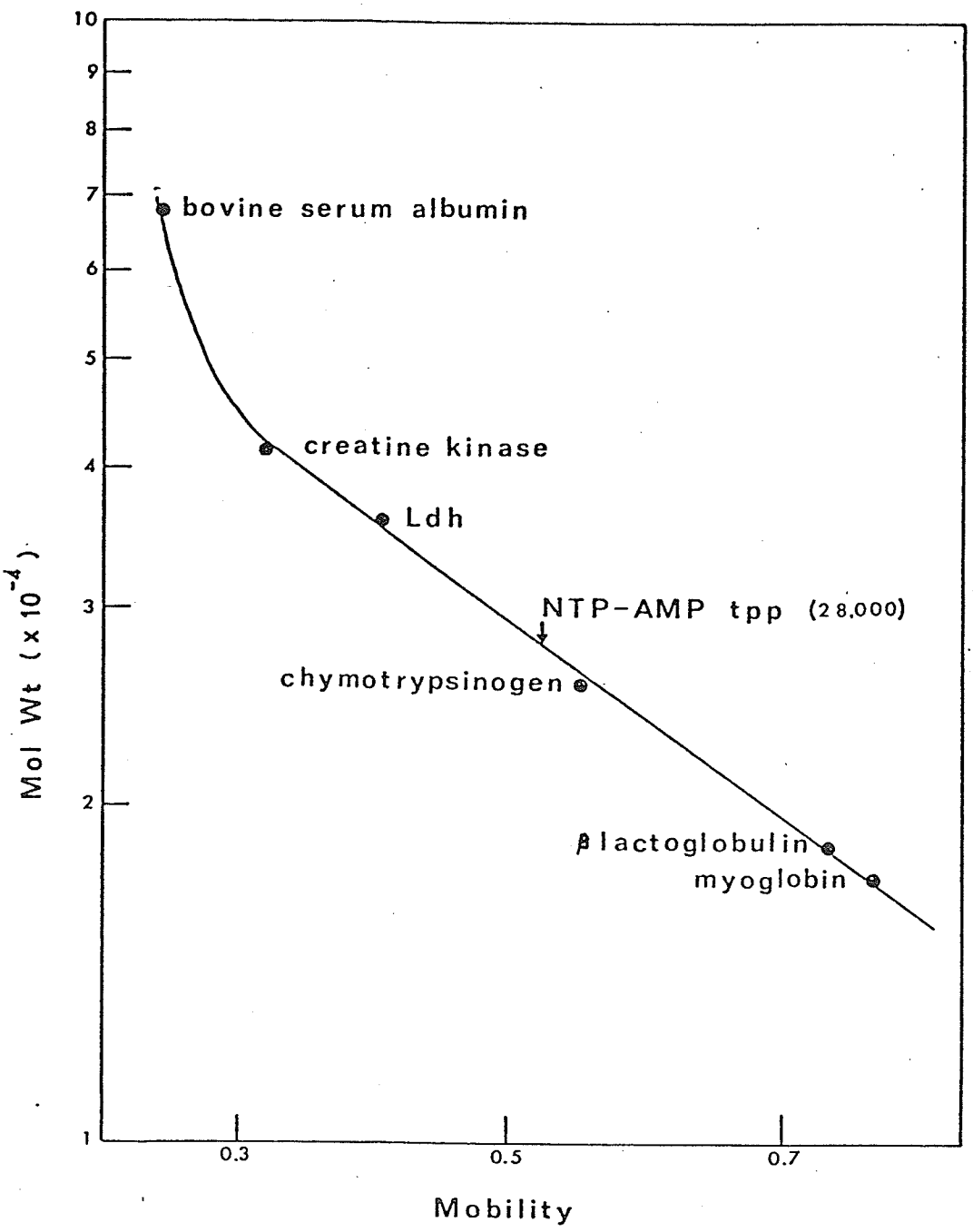


Figure 15. A calibration plot for the molecular weight determination of NTP-AMP tpp from SDS gel electrophoresis. The mobility is the (protein distance x length of gel before stain) ÷ (length of gel after destaining x the distance of bromophenol blue).

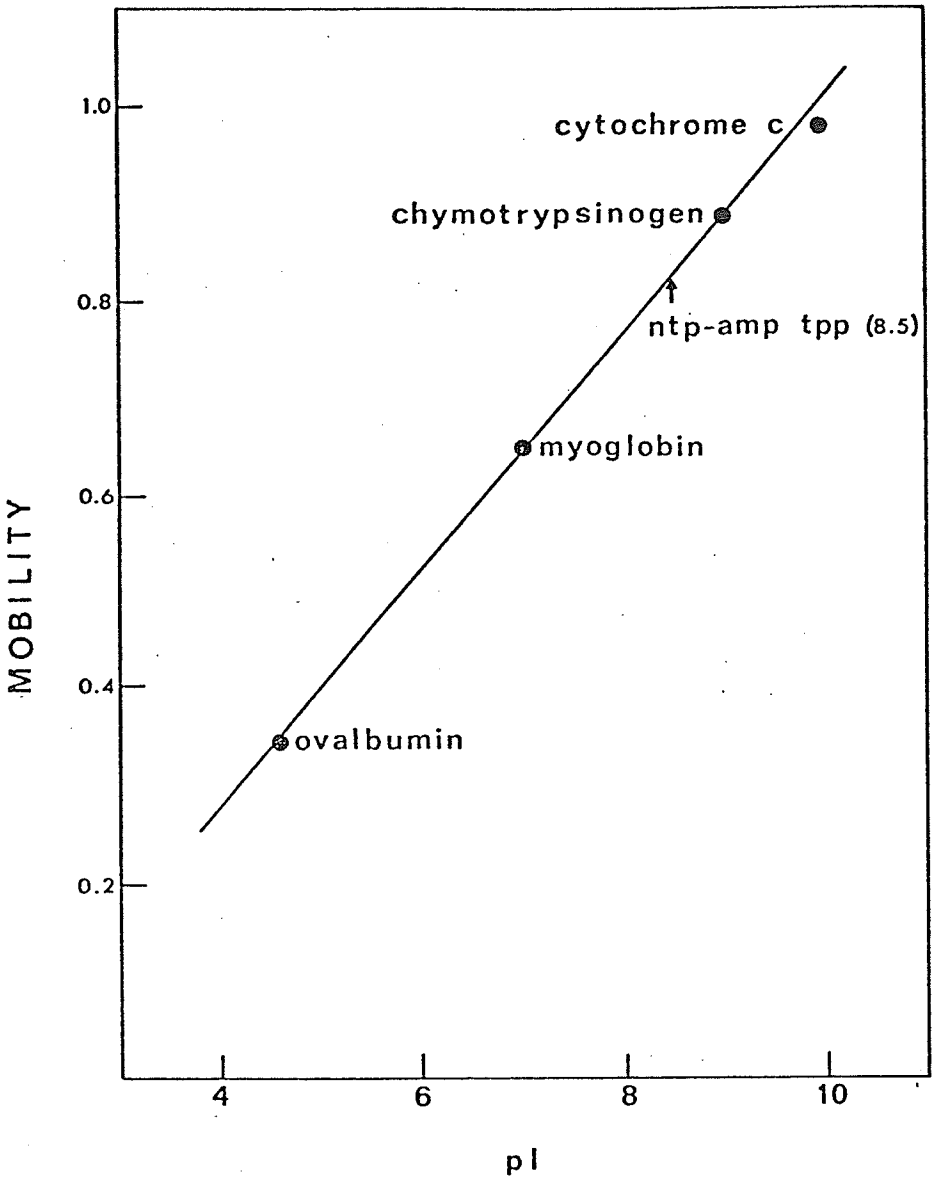


Figure 16. A calibration plot for the determination of the isoelectric point of NTP-AMP tpp by gel isoelectric focusing. The mobilities were calculated from the distances of the protein bands divided by the gel lengths.

which the pI of NTP-AMP tpp was obtained. It was found to be 8.5. Unfortunately whereas the temperature of the buffer surrounding the gels in this technique was easily measurable and probably corresponded to the temperature of the outside of the gels themselves it was impossible to measure the temperature in the centre of the gels. The exact temperature at which this isoelectric point applies is therefore not known.

b) Density Gradient Isoelectric Focusing

A more accurate determination of the isoelectric point of NTP-AMP tpp was made from isoelectric focusing in a thermostatically controlled sucrose density gradient system using a narrow range of ampholytes, from pH 8 to 11. Figure 17 shows the position of NTP-AMP tpp activity with respect to the pH gradient. From this plot an isoelectric point of 8.8 was estimated for 20°.

Unfortunately, because of the nature of the focusing process the ionic strength when this is complete is essentially zero and therefore very unfavourable to NTP-AMP tpp activity. The recoveries of NTP-AMP tpp from isoelectric focusing were consequently very low (usually about 5%).

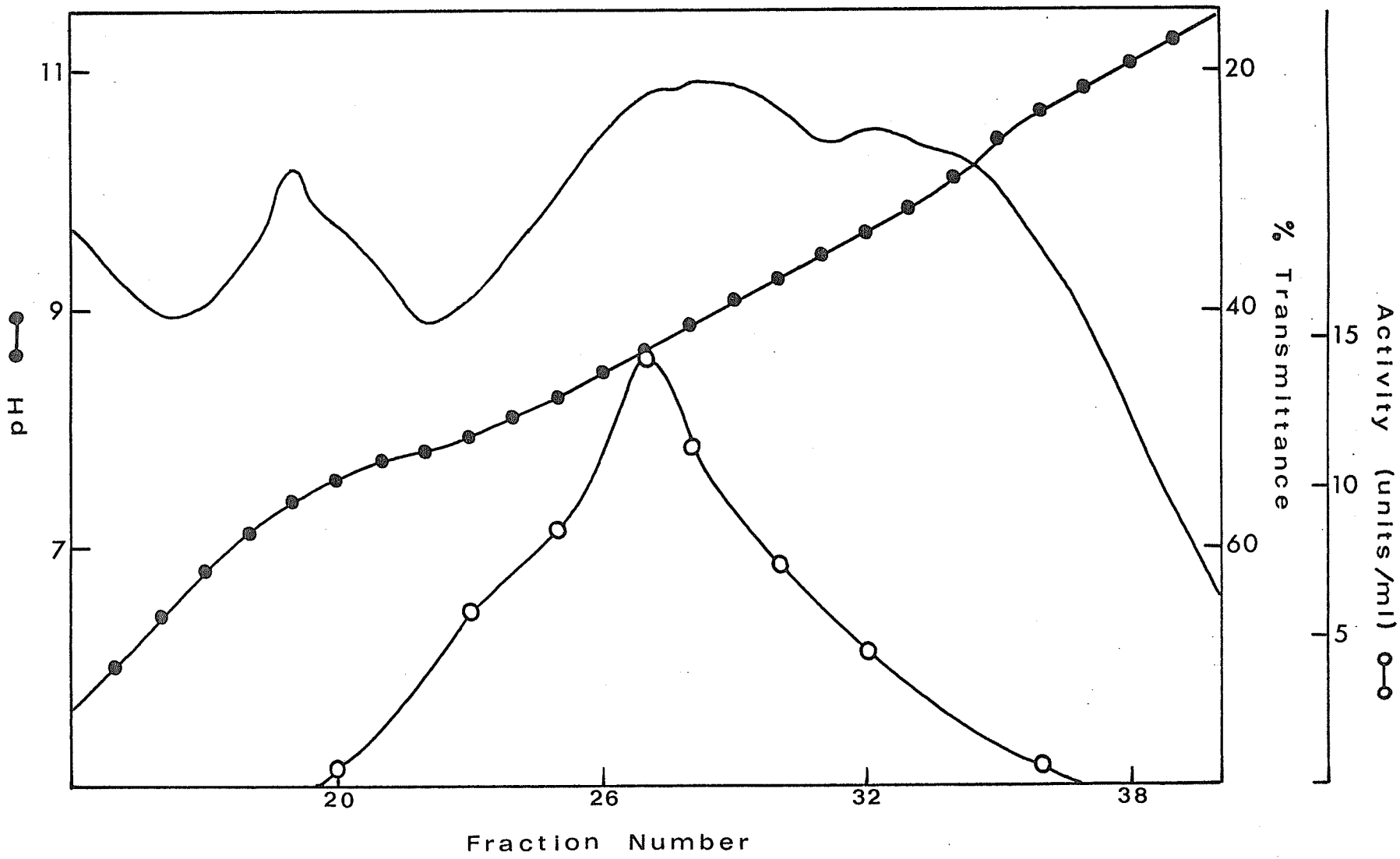


Figure 17. Sucrose density gradient isoelectric focusing of NTP-AMP tpp. This is the pattern obtained for the inner membrane enzyme. The % transmittance profile was obtained by an absorbance monitor. For further details see "Methods".

C. The Possible Existence of Two Forms of NTP-AMP tpp

In our initial studies of NTP-AMP tpp the three step extraction process had not yet been devised. This meant that we were working with essentially the enzyme from the inner membrane compartment of mitochondria only. This enzyme was extremely stable at room temperature and high ionic strengths. Chromatographies such as the ones involving Celite or Sephadex G-75 therefore invariably produced 90-100% recoveries of the enzyme. However, after introduction of the new extraction production, we began to notice, what seemed to be changes in the enzyme's stability properties. The yields of the once so successful chromatographies began dropping to 80% or lower.

Later as we obtained purer enzyme certain evidence seemed to indicate that, in fact, what we may have been isolating since the new extraction procedure was two forms of NTP-AMP tpp. Differences in their stability properties would explain the lowered recoveries on chromatographies.

The two "forms" were first seen in the SP-Sephadex chromatography. Two overlapping peaks of NTP-AMP tpp activity were routinely eluted from such a column (see Figure 4, p. 33). As shown in Figure 18 there also sometimes appeared to be two NTP-AMP tpp peaks in the Sephadex G-75 superfine chromatography. SDS and pH 4.3 cathodic disc gel electrophoresis of this material (Figure 19) both showed two bands of very similar mobility and approximately equal concentrations. Their molecular weights were estimated from the SDS gel electrophoresis to be 28,500 and 26,200.

Since the appearance of the "second form" coincided with the introduction of the digitonin treatment for obtaining the enzyme it seemed

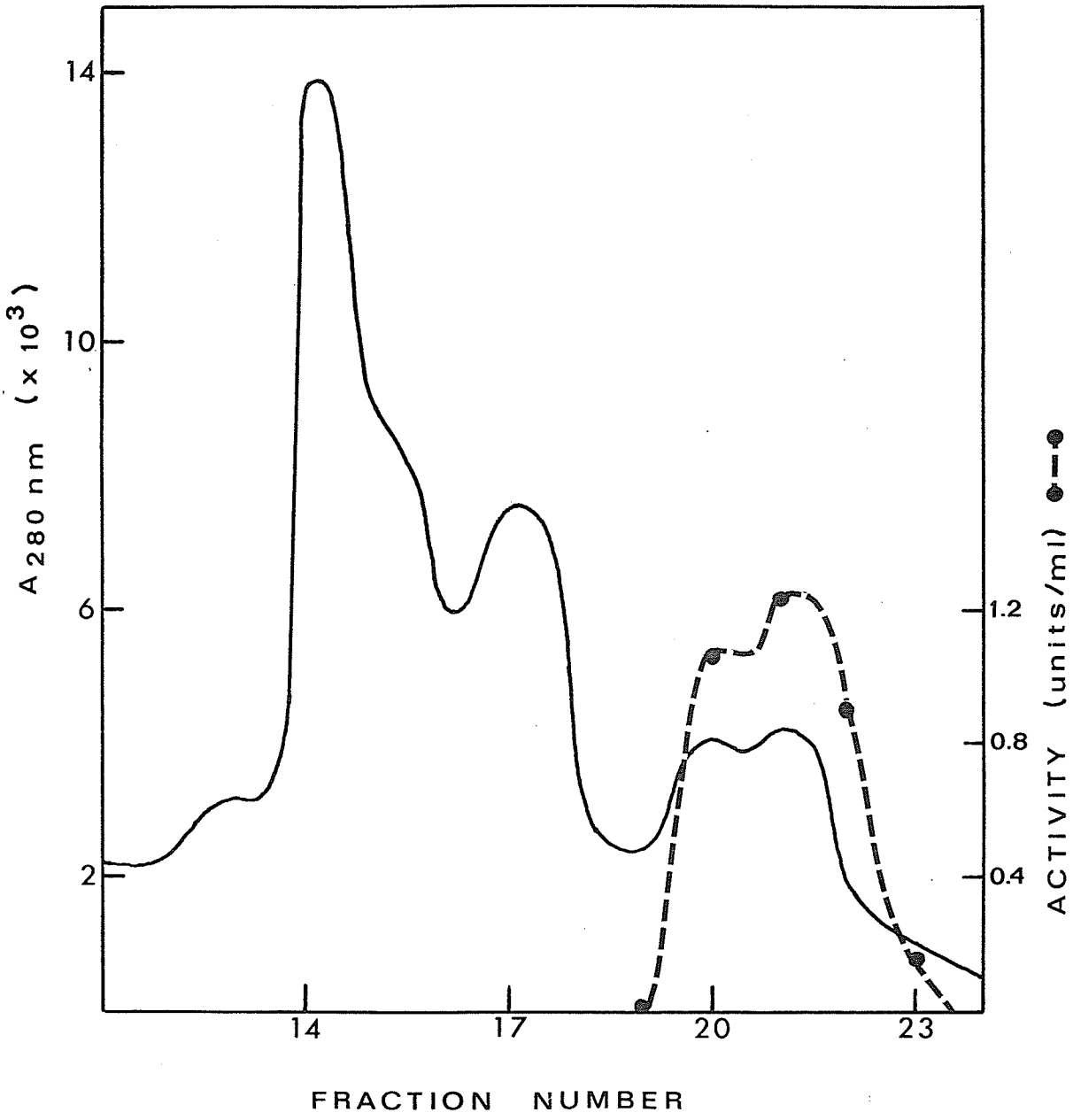
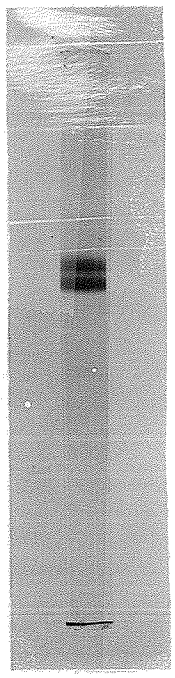


Figure 18. Sephadex G-75 superfine chromatography showing two peaks of NTP-AMP tpp activity. This chromatography was performed on a 2.5 x 100 cm. column, eluted at 26 ml/hr. and collecting 10 ml fractions. The absorbance at 280 nm was obtained with an absorbance monitor.

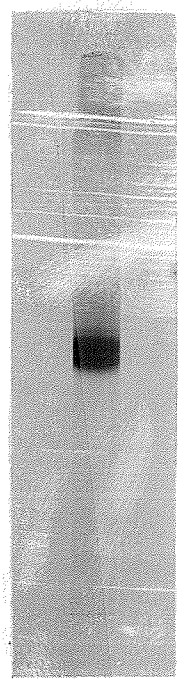
Figure 19. SDS gel electrophoresis and pH 4.3 cathodic disc gel electrophoresis of the two "forms" of NTP-AMP tpp.

a) SDS Gel Electrophoresis



← bromophenol blue

b) pH 4.3 Cathodic Disc Gel Electrophoresis



← cytochrome c

possible that the two forms could correspond to a newly introduced outer membrane NTP-AMP tpp and the original inner membrane enzyme. This would be similar to the case of carnitine palmitoyltransferase where one form of this enzyme is thought to be loosely associated with the outside of the inner membrane of mitochondria and another form bound tightly to the inner membrane (97).

To investigate this possibility of having an inner and outer enzyme, the isoelectric points of the enzyme released by digitonin treatment (outer enzyme) and the enzyme released by sonication (inner enzyme) were determined separately by sucrose density gradient isoelectric focusing. The pI's were found to be very close. Isoelectric points of 8.85 and 8.70 were found for the outer and inner membrane enzymes respectively. Also, neither the enzyme from the digitonin extract nor the enzyme released by sonication were found to be activated by dithiothreitol. It appears then that even if there are outer and inner membrane forms of NTP-AMP tpp, they are quite similar.

It is also possible, of course, that the second "form" is an artifact of the purification process. Purification of the two forms and a comparison of their physical and kinetic properties will be necessary, however, before it can be properly concluded whether the two forms of NTP-AMP tpp exist and if so, whether they are true isoenzymes or whether they are pseudoisoenzymes resulting from structural alterations produced by the isolation procedure.

V. DISCUSSION

A much improved purification scheme has been devised for NTP-AMP tpp. Previous attempts to purify this enzyme have been characterized by overall low yields, poor purifications, and the failure of even standard purification techniques to prove useful. The best so far reported purification of the enzyme was that achieved by Albrecht (17). He succeeded in purifying the bovine heart enzyme 40-fold and obtaining enzyme with a specific activity of 136 units/mg. Our scheme has far surpassed his, producing enzyme close to homogeneity, over 400-fold purified and with a specific activity over three times greater than his reported best value.

A careful investigation of the general characteristics of NTP-AMP tpp has also been undertaken. The enzyme was found to be a monomeric, low molecular weight protein with a basic isoelectric point. At least 24% of the total NTP-AMP tpp in bovine mitochondria was shown to be located in the outer membrane region with the remainder in the inner membrane compartment. Evidence was found which indicated the possible existence of two forms of NTP-AMP tpp. In general NTP-AMP tpp was shown to have some rather unusual stability properties. It was found to be stable at room temperatures or higher and high ionic strengths but labile at cooler temperatures (5° or less) and low ionic strengths.

Among other things I feel this careful investigation of the enzyme's characteristics has given us better insight into the unique properties which have made this enzyme so difficult to purify. Firstly, its extreme lability at low ionic strengths and rather basic isoelectric point places severe limits on the applications of standard ion-exchange or

adsorbent techniques for purification. Its pI of almost 9 restricts any binding of the enzyme to a cation exchanger. The high pH required to bind it to an anion exchanger would result in severe denaturation of the enzyme as can be seen from its pH stability curves. Secondly in any attempt to bind either the enzyme or impurities to an ion-exchanger or adsorbent the ionic strengths must be kept moderately high in order to preserve the NTP-AMP tpp activity. This would result in strong inhibition of the binding process and necessitate the use of a very strong exchanger or adsorbent as well as drastic pH conditions to compensate for it. Our SP-Sephadex purification step illustrates that even this does not work that successfully for the enzyme.

The relatively low concentration of enzyme in beef heart mitochondria would explain the overall low amounts of enzyme usually obtained despite fairly high recoveries throughout the purification process. If the specific activity of homogeneous NTP-AMP tpp is taken to be approximately 500 then according to the yields we generally obtained, this should correspond to 6.68 mg of enzyme extractable from our standard preparation of mitochondria from two hearts. However, this is only 0.2% of the total extractable protein usually obtained from the same quantity. This point is also emphasized on inspection of the SDS gel electrophoresis patterns obtained throughout the preparation. The NTP-AMP tpp band can be barely distinguished in the gel of the initial extracts which contains over 20 other bands.

If in fact two forms of mitochondrial NTP-AMP tpp exist this poses an additional difficulty in purification. It appears from our investigations that the two forms differ very little in their major

physical properties of molecular weight and isoelectric point. Unless of course they correspond to the outer and inner membrane compartments, a complete separation of them should be quite difficult. However, we have seen indications that the new form may not share all the same stability properties of the inner membrane enzyme. It may be that by performing a purification which uses conditions favouring one form that the other form is sacrificed.

Knowing some of these properties has allowed us, during the search for purification techniques, to chose methods which either try to overcome the enzyme's unfavourable properties or take advantage of its unusual stability. The unorthodox SP-Sephadex chromatography was used in an attempt to find a technique based on charge which overcame the enzyme's instability at low ionic strengths. The ammonium sulfate gradient solubilization and gel filtration steps were designed to make full use of NTP-AMP tpp's high ionic strength stability and of course the heat denaturation step made use of its heat stability.

Throughout the course of this investigation we have been careful to eliminate possible confusion of adenylate kinase with NTP-AMP tpp. That the two are evidently different can be seen by a comparison of a few of their properties (see Table 6 p.72). However, the enzymes do share one common feature--they both can use as substrates either ATP or GTP although to much different degrees. Adenylate kinase in general catalyzes the GTP-AMP reaction to a small extent but much less effectively than the ATP-AMP reaction. We have found that NTP-AMP tpp seems to catalyze the reaction with ATP about 15% as effectively as with GTP. In cases then where it was possible we were mistaking adenylate

kinase for NTP-AMP tpp we have been careful to check that the enzyme activity obtained in the assay with GTP as substrate exceeds that obtained with ATP as substrate.

We have also noticed several instances in the literature where it appears that the authors may have made the opposite mistake--confused NTP-AMP tpp with adenylate kinase. For example, Russell et al.(98) have separated and investigated the properties of numerous so called adenylate kinase isoenzymes from human organs. However, their assay for adenylate kinase is based only on the ATP-AMP reaction and they do not check for GTP-AMP activity. It may easily be--and the properties of some of their isoenzymes support this--that some of their adenylate kinase isoenzymes are in fact NTP-AMP tpp.

During the course of investigation we have tried where possible to compare our results with those obtained by other workers, especially Albrecht. Table 5 lists the properties of NTP-AMP tpp we have elucidated and compares them with those found by Albrecht.

In general the stability properties we determined for NTP-AMP tpp seem to agree with observations made by Albrecht. He did not carry out pH-stability studies but found a pH for optimum activity of 8.5 which is similar to the pH's of optimum stability that we found.

Although, Albrecht strangely did not determine an isoelectric point for NTP-AMP tpp he does claim to have run the enzyme on "poly-acrylamide gel electrophoresis". As he does not mention the technique used, it is assumed he meant the standard Davis and Ornstein (99) system of disc gel electrophoresis. If this is the case, and he is truly seeing a NTP-AMP tpp band, then its pI must be lower than pH 8.9, the running

TABLE 5

A Comparison of Our NTP-AMP tpp Properties

With Those Determined by Albrecht

Our Properties	Albrecht's Properties
1. Stability Properties:	
1) stable at high ionic strengths, not stabilized by AMP	1) stabilized by presence of ions or substrates
2) stable at high temperatures	2) heat stable
3) unstable at low ionic strengths, low temperatures, and when exposed to freezing or low protein concentrations	3) unstable when exposed to high dilutions, low ionic strengths, and freezing and thawing
4) pH of optimum stability: 7.5 (30°) 8.5 (5°)	4) pH of optimum activity: 8.5
2. Physical Properties	
1) isoelectric point: 8.8	1) pI much less than 8.9
2) molecular weight: 30,000	2) molecular weight: 52,000
3. Other Properties	
1) specific activity of pure enzyme about 500	1) claims enzyme with specific activity of 136 is $\geq$ 75% pure
2) NTP-AMP tpp located in both the inner and outer membrane compartments of mitochondria	2) enzyme bound only to inner membrane

pH of such a system. We have determined the isoelectric point to be about 8.8 and as would be expected have not been able to electrophorese NTP-AMP tpp in such a system.

We have determined by three systems of Sephadex G-75 gel filtration and SDS gel electrophoresis that NTP-AMP tpp is a monomer of 30,000 molecular weight. This is considerably lower than the molecular weight of 52,000 found by Albrecht. However, his technique for its determination casts some doubt on its validity. Firstly he used gel filtration on Sephadex G-200, certainly not the gel of optimum pore size for a molecular weight in the order of even 50,000. Secondly, he used only three marker proteins of molecular weights distant from the enzyme and appears to have performed the experiment only once.

Our estimation of the specific activity of the purified enzyme also differs from that predicted by Albrecht. Using his estimate of 75% or greater purity for his enzyme preparation, purified NTP-AMP tpp should have a specific activity of 180 which is considerably lower than the specific activity of 480 of our almost homogeneous enzyme. As mentioned previously, however, the gel electrophoresis upon which Albrecht's estimate of purity is based is already in doubt.

Finally, Albrecht claimed to have found no NTP-AMP tpp activity extractable from mitochondria by 75 mM PO_4^{-3} and concluded that all the enzyme was bound to the inner membrane. We have shown conclusively that over 20% of the enzyme from bovine heart mitochondria is located in the outer membrane compartment and should be extractable by PO_4^{-3}.

A comparison was also made between the basic properties of NTP-AMP tpp and those of the other nucleoside monophosphokinases. Such

a comparison is summarized in Table 6. A number of similarities are quite apparent. All the kinases seem to be monomeric with similar, low molecular weights. With a few exceptions they seem to show similar general stability properties. Their requirements for reducing agents show an interesting similarity. With adenylate kinase, dTMP kinase, and CMP-dCMP-UMP kinase, some enzymes show a requirement for sulfhydryl reducing agents where others do not. In the case of the latter two kinases the mammalian enzyme's require reducing agents whereas E. coli or Tetrahymena pyriformis enzymes do not. With adenylate kinase the cytoplasmic muscle enzymes appear to require reducing agent whereas the liver mitochondrial or yeast enzymes do not. For the other two monophophokinases so far no requirement for thiol compounds has been found. The isoelectric points investigated were limited, unfortunately. However, at least NTP-AMP tpp and adenylate kinase seem to have very similar isoelectric points. It is interesting to speculate that, because of their close similarity, the nucleoside monophosphokinases may have evolved from the same protein.

NTP-AMP tpp and the other nucleoside monophosphokinases differ very much in one respect--substrate specificity. NTP-AMP tpp, although very specific for AMP, has a broad specificity for the nucleoside triphosphate. The other kinases are generally very specific for one particular NMP and for ATP as the phosphate donor. It has been suggested that this specificity might enable the NTP-AMP transphophorylases to play an important role in the regulation of nucleotide triphosphate synthesis (100).

TABLE 6

A Comparison of NTP-AMP tpp Properties with Those of Other Nucleoside Monophosphokinases

Kinase	Source	Mol. Wt.	pI	Requirement for Thiols	Stability Properties
1. NTP-AMP tpp	bovine heart mitochondria	30,000	8.8	none	very heat and high ionic strength stable
2. Adenylate Kinase	rabbit muscle (32)	21,500	9.3	activated by thiols	heat and acid stable
	bovine liver mit. (20) rat liver (21,22) yeast (25)	21,500		not activated by thiols	
3. GMP Kinase	erythrocyte and Sarcoma 180 ascites cells (47)	18,500-24,000	4.9-5.8	none	in general stable
4. dTMP Kinase	mouse hepatoma (57)	35,000		requires thiols	unstable
	E.coli (56)			none	stable
5. CMP-dCMP-UMP	rat liver (62,63) calf thymus (56)	15,000		requires thiols	
	Tetrahymena pyriformis (60)			none	

If the function of the ATP-NMP kinases is regulation then what is that of NTP-AMP kinase? Some evidence has been presented which suggests that at least the inner membrane enzyme is connected with the succinyl thiokinase reaction:

$$\alpha\text{Ketoglutarate} + NAD^+ + CoASH \rightleftharpoons \text{Succinyl-CoA} + CO_2 + NADH + H^+$$

$$\text{Succinyl-CoA} + Pi + GDP \overset{\text{succinyl thiokinase}}{\rightleftharpoons} \text{Succinate} + GTP + CoASH$$

Tager et al. (101) have suggested the following possible fates for the GTP produced in this reaction:

1. Nucleoside Diphosphokinase Reaction

Nucleoside diphosphokinase could catalyze the reaction of GTP with ADP to form ATP and thus regenerate GDP:

$$GTP + ADP \rightleftharpoons GDP + ATP$$

2. NTP-AMP tpp Reaction

$$GTP + AMP \rightleftharpoons GDP + ADP$$

3. Fatty Acid Activation

A liver mitochondrial enzyme under certain conditions has been shown to catalyze a GTP-linked activation of fatty acids (102):

$$\text{Fatty acid} + GTP + CoA \rightleftharpoons \text{Fatty acyl-CoA} + GDP + Pi$$

4. Phosphoenolpyruvate Synthesis

In mitochondria from liver and kidney PEP may be formed from oxaloacetate by the phosphopyruvate carboxylase reaction:

$$GTP + \text{oxaloacetate} \rightleftharpoons GDP + PEP + CO_2$$

5. Mitochondrial RNA and Protein Synthesis

Of these, the first two reactions are likely the most important. Of these two kinases it has been suggested by Heldt and Schwalbach (15) that the NTP-AMP tpp in rat liver anyway plays a more important role. In studies of the anaerobic dismutation of ∝ketoglutarate in the presence of oligomycin and [^{32}P] phosphate, they showed that [^{32}P] ADP rather than [^{32}P] ATP is the main product of the reaction.

Another consideration in the relative importance of NTP-AMP tpp and nucleoside diphosphokinase in connection with the succinyl thiokinase reaction is their intramitochondrial location. Approximately 70% of NTP-AMP tpp has been shown to be located in the inner membrane region of mitochondria where the succinyl thiokinase reaction occurs. On the other hand, only a small portion (5% or less) of the nucleoside diphosphate kinase has been found in this area (103), the remaining being located in outer membrane region. Because of the known impermeability of the inner membrane of mitochondria it seems more likely that NTP-AMP tpp rather than nucleoside diphosphokinase would make the greater contribution to the regeneration of GDP.

If outer and inner membrane forms of NTP-AMP tpp exist then it is interesting to contemplate what their role might be. The only other situation, so far reported, which is similar to this is the case of two forms of carnitine palmityltransferase in mitochondria. One form was found to be losely bound to the external surface of the inner membrane while the second form was firmly bound to the inner membrane. They have been shown to have different isoelectric points , substrate specificities, and sensitivities to inhibition by 2-bromopalmityl-CoA (104). It has been proposed that these two forms work together to transport long chain acyl

groups across the mitochondrial inner membrane for fatty acid oxidation (97). For example, carnitine palmityltransferase on the outer side of the inner membrane would catalyze the formation of palmityl-carnitine from palmityl-CoA and carnitine. It is thought that the inner membrane carnitine palmityltransferase might be vectorially oriented within the inner membrane so that the palmityl-carnitine is accessible to it. The transferase would then catalyze the formation of palmityl-CoA from the palmityl-carnitine and intramitochondrial CoA, making it available for β-oxidation.

In analogy to this , the two forms of NTP-AMP tpp might function in transporting GTP, in effect, across the normally impermeable inner membrane by coupling the high energy phosphate derived from it to adenosine monophosphate. If the GTP in a sense is to enter the inner membrane region then one can envision a situation where the outer membrane NTP-AMP tpp is located on the outer surface of the inner membrane in close proximity to the adenine nucleotide translocase. It could catalyze the transfer of a high energy phosphate group from GTP to AMP forming ADP which could be easily transported across the inner membrane by the translocase. On the inner side of the inner membrane the second NTP-AMP tpp could then catalyze the transfer of phosphate from the transported ADP to GDP, forming GTP again. Of course, this is highly speculative and a great deal more will have to be known about the two forms before any definite function can be proposed.

Overall then for NTP-AMP tpp as a whole much remains to be investigated. Hopefully the improved purification scheme described here might lead to the production of large quantities of homogeneous enzyme

which would enable careful kinetic analyses, studies of the enzyme's physical characteristics such as amino acid composition, and investigations into the mechanism by which it acts. Separation, purification, and investigation of the two "forms" will be required to determine if they are actually isoenzymes and what their possible function might be.

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