ENZYMIC SYNTHESIS OF NICOTINAMIDE ADENINE DINUCLEOTIDE AND RELATED COMPOUNDS IN THE PRESENCE OF ATP:NMN ADENYLYLTRANSFERASE.

by

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Abbreviations

AMP	adenosine monophosphate
ATP	adenosine triphosphate
8 aza GTP	8 aza guanosine triphosphate
CMP	cytosine monophosphate
CM cellulose	carboxymethyl cellulose
DEAE cellulose	diethylamino ethyl cellulose
deoxy AMP	2' deoxy adenosine monophosphate
deoxy ADP	2' deoxy adenosine diphosphate
deoxy ATP	2' deoxy adenosine triphosphate
deoxy GTP	2' deoxy guanosine triphosphate
deoxy NAD	P^1-nicotinamide riboside-5' P^2-deoxy adenosine-5' pyrophosphate
EDTA	ethylene diamine tetra acetic acid
FAD	flavine adenine dinucleotide
GTP	guanosine triphosphate
IMP	inosine monophosphate
ITP	inosine triphosphate
NAD	nicotinamide adenine dinucleotide
NMN	nicotinamide mononucleotide
Tris	tris (hydroxymethyl) amino methane
TTP	thymidine triphosphate
UTP	uridine triphosphate

Summary

The summary of this thesis is on page 187.

Acknowledgements

I wish to thank my supervisors, Professor R. K. Morton and Dr. M. R. Atkinson, for their guidance during my course of study. The financial assistance of a Commonwealth Post-Graduate Studentship (1960) and an Australian Dairy Produce Board Senior Post-Graduate Studentship (1961 and 1962) is gratefully acknowledged.

Declaration

I hereby declare that the work presented in this thesis has been performed by myself, except where otherwise stated in the text, and that it has not been submitted in any previous application for a degree.

INTRODUCTION

The enzyme ATP:NMN adenylyltransferase (Enzyme Commission number: 2.7.7.1) catalyses the reaction of adenosine triphosphate with nicotinamide mononucleotide to give nicotinamide adenine dinucleotide and inorganic pyrophosphate (Figure 1 and equation 1).

(1) ATP + NMN $\rightleftharpoons$ NAD + pyrophosphate

The equilibrium constant for this reaction is approximately 0.45 (Kornberg, 1950), so that the reaction can be readily demonstrated in both directions. It was first described by Kornberg (1948, 1950), who partially purified the corresponding enzyme from both yeast and pig liver. The enzyme has subsequently been found in many animal tissues (see Morton, 1961) where it appears to be localised in the cell nucleus (Hogeboom and Schneider, 1952; Branster and Morton, 1956).

More recently it has been found that the enzyme from pig liver will also catalyse nucleotidyl transfer from ATP to nicotinic acid mononucleotide, yielding nicotinic acid adenine dinucleotide and pyrophosphate (shown in parenthesis in Figure 1); this reaction has been postulated as a step in the biosynthesis of NAD (Preiss and Handler, 1958). The enzyme from liver is apparently quite distinct from those found in Escherichia coli and in human erythrocytes which are specific for nicotinic acid nucleotide and its adenylyl derivatives (Fig.1) (Imsande, 1961; Preiss and Handler, 1958a).

These enzymes are members of a group which catalyse the reaction of a nucleoside triphosphate with an orthophosphate ester of an alcohol,

1 (a)

Fig. 1

The reaction catalysed by ATP:NMN adenylyltransferase.

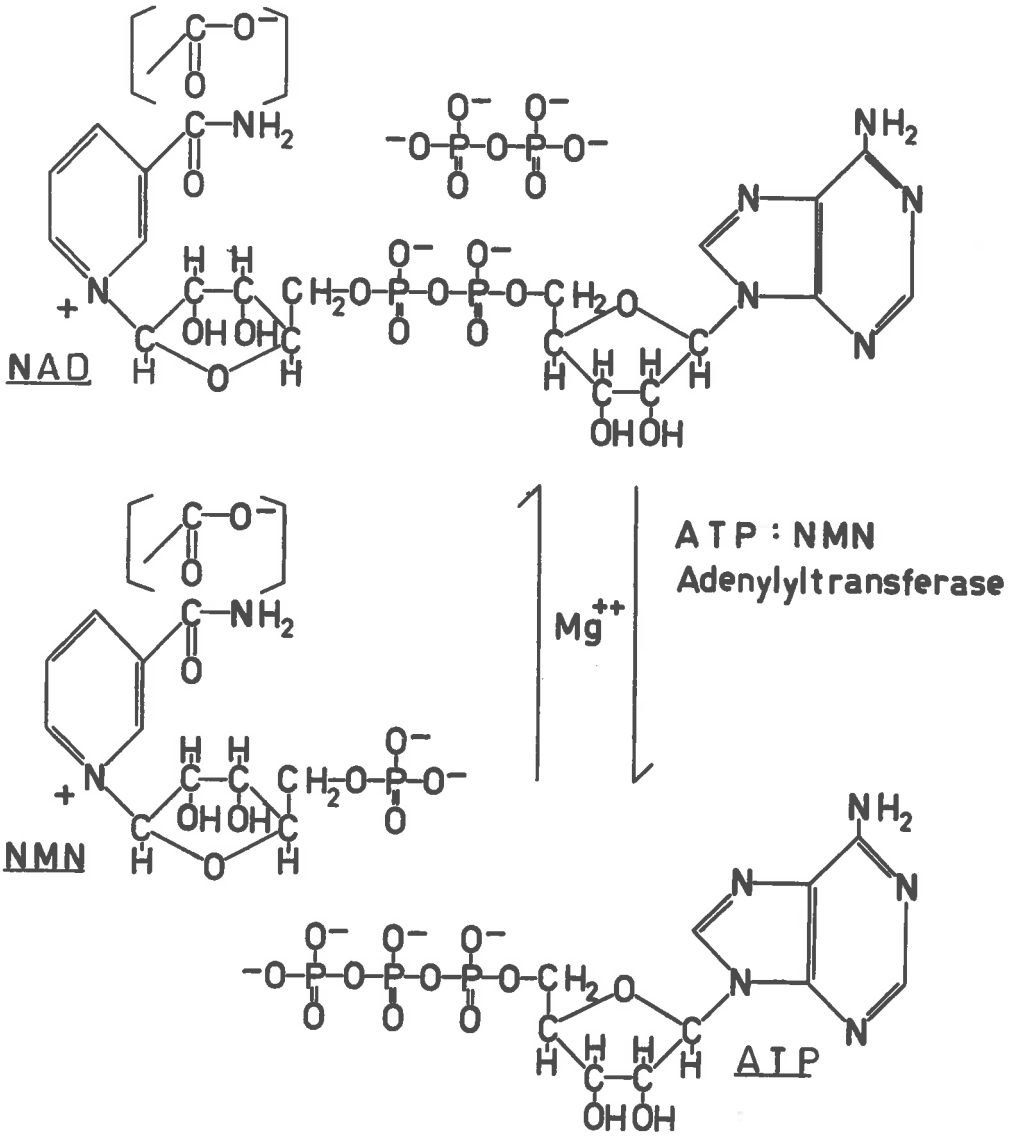
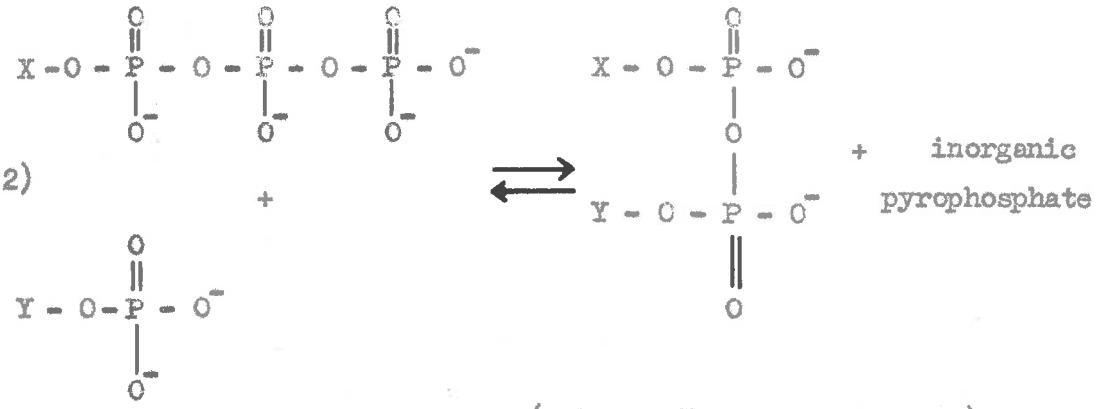


Fig.1

giving rise to a pyrophosphate diester and inorganic pyrophosphate (equation 2).

(2)



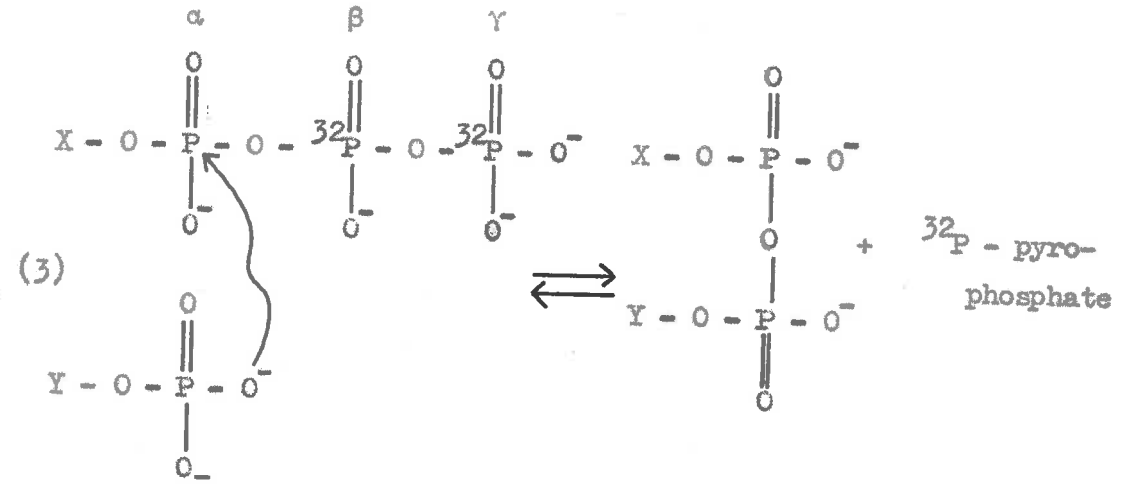
(where XOH is a nucleoside)

In this thesis the term 'pyrophosphorylase' is restricted to this class of enzymes; the related enzymes which catalyse pyrophosphorolysis of orthophosphate diesters or of C-N bonds will not be considered. Twenty four of these pyrophosphorylases, catalysing reactions of the type shown in equation (2), have been described. There are several for each of the four naturally occurring ribonucleoside triphosphates (UTP, CTP, GTP and ATP) and two that catalyse reactions involving the deoxyribonucleoside triphosphate, TTP (Table V - 13).

All these pyrophosphorylases are thought to be involved in processes of synthesis within the living cell. A sugar phosphate always acts as the nucleotidyl acceptor in the reactions catalysed by the 9 pyrophosphorylases utilizing UTP as the nucleoside triphosphate. The pyrophosphate diesters so formed are further utilized for polysaccharide synthesis (Roseman, 1959; Strominger and Smith, 1959). Of the 5 pyrophosphorylases using CTP, two are involved in the synthesis

of pyrophosphate diesters which act as precursors for teichoic acids of the cell wall of microorganisms (Shaw, 1962) and two in the biosynthesis of phospholipid precursors (Kennedy and Weiss, 1956). Pyrophosphorylases which catalyse guanylyl transfer from GTP give products which are thought to be involved in polysaccharide formation (Ginsberg, O'Brien and Hall, 1962), and those utilizing TTP form dinucleotides which may also be involved in polysaccharide synthesis (Kornfeld and Glaser, 1961). The pyrophosphorylases involving ATP are implicated in polysaccharide synthesis in plants (Recondo and Leloir, 1961), and in the synthesis of such cofactors as coenzyme A Hoagland and Novelli, 1954), FAD (Schrecker and Kornberg, 1950; DeLuca and Kaplan, 1958) and NAD (Kornberg, 1950; Preiss and Handler, 1958; Imsande, 1961).

It is clear that this class of enzymes has an important part to play metabolically, and that its members should have many features in common in view of the type of reaction they catalyse. A little is known of the actual mechanism of the reaction. For the reaction catalysed by ATP:NMN adenyl/transferase from pig liver, inorganic ^{32}P pyrophosphate was incorporated into the β and γ positions of ATP on pyrophosphorolysis of NAD (Kornberg and Pricer, 1951). The adenylyl transfer may be formulated as a nucleophilic attack of the mononucleotide on the α-phosphorus atom of ATP (equation 3).



This mechanism is also indicated for the reactions catalysed by CTP:choline phosphate cytidylyltransferase and CTP:ethanolamine phosphate cytidylyltransferase, since, when the mononucleotide concerned, labelled with ^{32}P, is incubated with CTP and transferase, the label appears in the synthesised dinucleotide (Kennedy and Weiss, 1956). Radioactive pyrophosphate was not incorporated into the triphosphate in the presence of enzyme unless the monophosphate ester was present and it has been postulated that a nucleotidyl-enzyme complex is not formed by ATP:NMN adenylyltransferase (Kornberg and Pricer, 1951), UTP:α-D-glucose-1-phosphate uridyltransferase (Munch-Petersen, 1955, 1957) and the uridylyl transferases of mung bean preparations (Neufeld et al., 1957).

In general the properties of the pyrophosphorylases have not been studied to any great extent, and in no case has a member of this important group been obtained in a homogeneous form. The present thesis deals with a study of one member of this class of enzymes, ATP:NMN adenylyltransferase from pig liver. The investigations have

been aimed at furthering our knowledge of the mechanism of action of this enzyme. Wherever possible in this report, the findings with this enzyme are discussed in relation to possible similarities with other pyrophosphorylases.

There are three main ways of studying the mechanism by which an enzyme catalyses a reaction. All are dependent on unusual characteristics not found with other catalysts. The first of these is a kinetic approach. In general, enzymes can bring about a reaction at a higher rate than can other known catalysts (Laidler, 1958, p.164). In addition, the rate laws for enzymic catalysis are different from those, for example, for acid-base catalysis, where the rate of reaction increases indefinitely as the substrate concentration is increased. The kinetics of enzymic reactions are usually governed by the Michaelis-Menten law (Michaelis and Menten, 1913), which implies that an increase in the concentration of the substrate above a certain level does not bring about a further increase in the rate of reaction. The law is based on the hypothesis that the enzyme forms a complex with its substrate, and that this complex then breaks down to give the products of the reaction. There is now some evidence that such complexes are in fact formed (Chance, 1943, 1949).

The velocity of an enzymic reaction is given by equation (4),

$$v = \frac{V_{max}}{1 + \frac{K_m}{S}} \qquad (4)$$

where v is the rate of reaction at substrate concentration S, V_{max} is the theoretical limiting velocity at high substrate concentration and K_m is a constant termed the Michaelis constant. In practical

terms this is the substrate concentration for which $v = V_{max}/2$. In some cases the Michaelis constant is thought to be a measure of the dissociation constant for the enzyme-substrate complex and thus can give an indication of the affinity of the substrate for the enzyme, However, this assumption has to be treated with caution, as Briggs and Haldane (1925) have shown that the constant may contain a kinetic element and so not be a true measure of this affinity (Dixon and Webb, 1958, p. 96).

A second approach to the elucidation of mechanism is to study the effect of hydrogen ion concentration on the rate of the enzymic reaction. In the majority of cases the rate is found to pass through a maximum as the pH is varied. A kinetic study of these effects can lead to a knowledge of the state of ionization of groups involved in the catalysis (Dixon and Webb, 1958, p. 120; Alberty, 1956; Laidler, 1958, p. 117). Such an investigation has been carried out in the ATP:NMN adenylyltransferase reaction (Atkinson, Jackson and Morton, 1961; Jackson, 1960). From a statistical analysis of the variation of K_m and V_{max} for NMN with pH, it was deduced that two dissociating groups, with pK values of 5.76 and 9.97, participated in the reaction, and that these groups are involved in the binding of NMN to the enzyme (or enzyme-ATP-magnesium complex) and not in the breakdown of the enzyme-substrate complex. One of these groups is thought to be the phosphoryl group of NMN, where the doubly dissociated ionic species would have a greater ability to carry out a nucleophilic attack on the α-phosphorus atom of ATP (equation 3). The other dissociating group was thought to be in the enzyme or the enzyme-ATP-magnesium complex (Atkinson, Jackson and Morton, 1961).

A study of the specificity of an enzyme provides a third way of learning more about enzymic mechanisms. The 'lock and key' hypothesis of Fischer (1894), who sought to explain the extraordinary selectivity of the chemistry of living cells, forms the basis of more recent ideas on the specificity of enzymes. Side by side with the growing conviction that the overall shape of a substrate molecule determined whether or not it would enter into a reaction catalysed by a particular enzyme, the concept of an active site (for a review see Koshland, 1960) in the enzyme was introduced; it was postulated that the substrate molecules are bound as a preliminary to the reaction which gives rise to the products. There are indications that the substrate molecules are bound to the active site by means of weak chemical forces such as hydrogen bonds and electrostatic attractions, and by nonlocalised dispersion forces (van der Waals forces) which arise from the complementary shape of substrate and active centre (for discussion of this point see Woolley, Hershey and Koehelick, 1962; Koshland, 1959; Dixon and Webb, 1958, p. 242). Thus a kinetic study of the rate with which substrate analogues can participate in an enzymic reaction may throw some light on the groups in the active centre and in the substrate molecule which are responsible for these attractive forces.

Most of the present investigation is concerned with third approach to the mechanism of action of ATP:NMN adenylyltransferase The aim of this work has been to examine the specificity of the enzyme from pig liver with respect to compounds related to the two substrates

ATP and NMN, and to interpret the results in terms of the above theories. The transferase requires a metal ion as cofactor in the reaction; magnesium and manganese ions will satisfy this requirement (Kornberg, 1950). To gain a better knowledge of the function of this metal ion in the reaction a kinetic study of the specificity of the enzyme with regard to a number of divalent metal ions has been attempted.

These investigations could have several applications. The substrate specificity pattern may indicate the requirements for a molecule to be an inhibitor of the transfer reaction. Such an inhibitor may be useful in chemotherapy (Morton, 1958). Secondly, the transferase from pig liver has been shown to be capable of using either NMN or nicotinic acid mononucleotide as the nucleotidyl acceptor (Preiss and Handler, 1958), and it has been suggested that nicotinic acid mononucleotide is the natural substrate <u>in vivo</u> (Preiss and Handler, 1958; Imsande and Handler, 1961). Quantitative studies of the specificity of the reaction, with regard to the mononucleotide reactant, may indicate the relative importance of NMN and nicotinic acid nucleotide as biological precursors of NAD.

Recent work indicates that there is an inverse relationship between the rates of NAD synthesis and ribonucleic acid synthesis in cell nuclei (Revel and Mandel, 1962). This may result from depletion of the pool of nucleoside triphosphates through nucleotidyl transfer to pyridine nucleotides, and information about the specificity of the nuclear enzyme studied here is needed for any evaluation of its function in this inhibition of nucleic acid biosynthesis.

The report that follows also includes a section on the purification of ATP:NMN adenylyltransferase from pig liver. This work was carried out in an attempt to obtain enzyme which was free of interfering activities and thus suitable for the kinetic experiments reported here.

I

Purification of ATP:NMN Adenylyltransferase from Pig Liver

In this section procedures for the extraction of ATP:NMN adenylyltransferase from pig liver acetone powder or the 'nuclear' fraction from pig liver cells are described. A brief description is given of attempts to purify the transferase from sodium chloride extracts of pig liver nuclei. This is followed by an account of a reproducible method of purification of the enzyme from phosphate extracts of the 'nuclear' fraction. Procedures for further purification are then described in some detail and finally an appraisal of the purity of the preparations so obtained is discussed in the light of their behaviour during starch gel electrophoresis.

A. Materials

Yeast alcohol dehydrogenase. A crystalline suspension was purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Suitable dilutions (see below) were made in 0.01 M-sodium phosphate, pH 7.5.

Diaphorase (dihydrolipoamide dehydrogenase). The enzyme was purified from pig heart as described by Massey, Gibson and Veeger (1960).

Calcium phosphate gel. Unless otherwise stated, the gel was prepared according to the method of Keilin and Hartree (1938).

Hydrolysed starch was obtained from the Connaught Medical Research Laboratories, Toronto, Canada (lot 140).

Ion exchange materials. DEAE cellulose was purchased from Pharmacia, Uppsala, Sweden, and carboxymethyl cellulose from Biorad Laboratories, Richmond, California. The Amberlite CG-50 used was of 100-200 mesh (type I).

Glycylglycine was obtained from British Drug Houses Ltd., Poole, Dorset (laboratory reagent grade).

ATP. Material of the purity described by Atkinson, Burton and Morton (1961 b) was from Sigma Chemical Co., St. Louis, Mo., U.S.A.

NMN. The mononucleotide was prepared as described by Atkinson, Jackson and Morton (1961).

Nitroblue tetrazolium. G. T. Gurr Ltd., London supplied this compound.

Detergent. The detergent used was 'Supreem' brand (formula 410) purchased from J. R. Alexander and Son Ltd., Adelaide. The main active constituent was probably sodium dodecylsulphate.

Urea. Analytical reagent grade urea was obtained from British Drug Houses, Ltd.

Acetone. British Drug Houses, Ltd. supplied the acetone, which was redistilled before use.

Inorganic reagents. These were analytical reagent grade (British Drug Houses, Ltd.)

B. Methods

Protein determination

In most cases the spectrophotometric method of Warburg and Christian (1942) was routinely used. In the descriptions of the purification of ATP:NMN adenylyltransferase given below, the ratio of absorption at 280 mμ to that at 260 mμ for the various preparations is indicated where possible. This permits estimation of the ratio of protein to nucleic acid to be made, and may be important in the present work, where the starting materials are derived from the cell nucleus, which is known to contain large amounts of nucleic acid. Warburg and Christian (1942) showed that the protein enolase had a ratio of 1.75, and nucleic acid a ratio of approximately 0.50. Where indicated, protein was estimated as described by Lowry et al. (1951).

Assay for ATP:NMN adenylyltransferase.

For routine estimation of the transferase during its purification a reaction mixture was employed containing NMN at a relatively low concentration, giving sub-optimal rates. A correction factor, based on a study of the effect of NMN concentration on the rate of transfer was used to calculate maximum velocities. The enzyme was incubated with 4 μmoles of ATP, 16 μmoles of $MgCl_2$, one μmole of NMN and 200 μmoles of glycylglycine, pH 7.6. The total volume of the incubation mixture was 1.0 ml., and was contained in a tube 10 cm. x 1 cm. internal diameter. After ten minutes at 37°, the reaction was stopped by the addition of 1.5 ml. of a 0.5 M-trichloroacetic acid solution and the resultant precipitate centrifuged to the bottom of the tube. Two ml. of the supernatant was pipetted into a spectrophotometer cell with a 4 cm. light path, followed by 3.0 ml. of a solution containing 0.835 M-ethanol - 0.417 M-glycine - 0.416 M-sodium hydroxide. After careful mixing of the resultant solution (pH about 9.5), NAD was determined from the increase in optical density at 340 mμ on addition of 0.02 ml. of a solution of yeast alcohol dehydrogenase containing 66 γ of protein. A millimolar extinction coefficient of 6.22 was used for reduced NAD (Horecker and Kornberg, 1948). After calculation of the total amount of NAD formed in the incubation mixture, this value was multiplied by a factor of 1.3 to allow for the sub-optimal concentration of NMN used during the assay. One unit of ATP:NMN adenylyltransferase was defined as the amount of enzyme which synthesises one μmole of NAD per minute under the above conditions, allowing for the low concentration of NMN. The specific

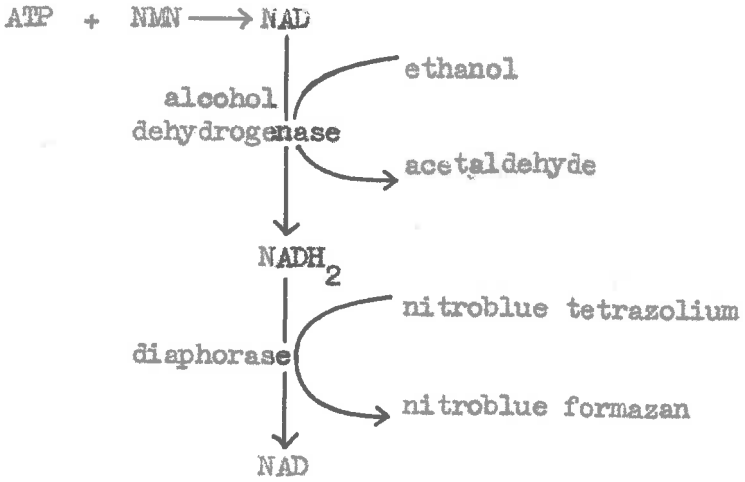
activity of the transferase preparation was expressed as units per mg. of protein.

Starch gel electrophoresis and the histochemical detection of ATP:NMN adenylyltransferase activity.

The starch gel electrophoresis of transferase preparations was carried out by the following modification of Poulik's (1957) method. Twelve grams of hydrolysed starch was suspended in 100 ml. of 0.09 M-Tris - 0.019 M-citrate - 0.5 mM-EDTA, pH 8.25. The mixture was heated over a gas flame. It became very viscous and with further heating the syrup became more mobile. At this stage it was degassed by means of a water pump for one to two minutes, and poured into a perspex tray with the dimensions 14.9 x 5 x 1.2 cm. A plastic cover was placed on top of the gel and the whole allowed to cool for about one hour. The gel was sliced level with the top of the tray, and the required number of slots made in it with 3 MM chromatography paper. 5 mm lengths of this paper were then soaked in the enzyme preparation (1 to 2% protein) and carefully placed in the slots. The gel was compressed a little from each end, the wicks (plastic sponge) put into position, and the wicks and gel covered with a piece of this plastic. The tray was than placed in the bath such that the two wicks dipped into the electrode vessels containing 0.3 M-borate (Na^+), pH 8.25. The bath was placed in a refrigerator at 2° and an electrical potential applied across the gel. Best results were obtained by applying 35 volts between the inner ends of the wicks for a period of 9 to 10 hours.

At the end of the run, when the borate front had moved a suitable distance towards the anode, the plastic cover and wicks were removed. The gel was then split horizontally with a thin wire and one half stained for protein with an amide black solution (0.672% in acetic acid-methanol-water). Excess amide black was removed by washing in acetic acid-methanol-water (1:5:5; vols) overnight on a rocking device. The other half of the gel was used to demonstrate areas of ATP:NMN adenylyltransferase activity. It is advisable to carry out the histochemical test for the transferase first in one half of the gel, and then to cut small incisions in the other half at positions of enzyme activity, before staining for protein. This corrects for shrinkage of the gel during the latter process.

The demonstration of areas of transferase activity on the starch gel was made possible by coupling together the ATP:NMN adenylyltransferase with yeast alcohol dehydrogenase and diaphorase. In the presence of ATP, NMN and ethanol this system converts the colourless nitroblue tetrazolium into an insoluble diformazan, which is purple in colour.



To carry out the test, 0.5 ml. each of the following two solutions were mixed : 8 mM-ATP - 32 mM-$MgCl_2$ - 2 mM-NMN - 400 mM-glycylglycine, pH 7.6 and 500 mM-ethanol - 200 mM-Tris, pH 9.5. 1.5 milligrams of nitrobluetetrazolium was dissolved in this mixture. Just before the experiment was carried out, 0.025 ml. of a 1% solution of pig heart diaphorase and 0.075 ml. of a solution of yeast alcohol dehydrogenase (containing approximately 250 γ of protein) were added. Whatman no 1 chromatography paper was cut to the approximate dimensions of the starch gel (15 cm. x 5 cm.) and soaked in this solution. The soaked paper was then carefully placed in contact with the cut surface of the gel. After ten minutes at room temperature, purple areas appeared in regions of ATP:NMN adenylyltransferase activity, and were fully developed after twenty minutes. The paper was quickly passed through a solution of glacial acetic acid-methanol-water (1:5:5; vols) and dried. The colour fades slowly in strong light. The use of paper to adsorb the precipitated diformazan and to carry substrates and enzymes provides a permanent record of the experiment. In carrying out this test it is advisable to avoid the use of a larger excess of yeast alcohol dehydrogenase than that indicated, as this leads to a faintly purple background. This phenomenon has been recorded by NachLas, Walker and Seligman (1958) when using yeast alcohol dehydrogenase and nitrobluetetrazolium to detect diaphorase in tissues. It is probable that the use of lactate dehydrogenase in combination with lactate in the above method would eliminate this background colour.

In some experiments starch gel electrophoresis was carried out

using apparatus and procedures similar to those described by Smithies (1959). However, the method described in detail above gave the best resolution of protein components in the case of ATP: NMN adenyltransferase preparations. Any major variation in methodology will be indicated in the text.

C. Results

(a) Extraction of ATP:NMN adenylyltransferase from an acetone powder of pig liver.

An acetone powder of pig liver was made by the method of Kornberg (1950) except that mincing of the liver was carried out in a power driven mincer. This enabled larger amounts of liver to be processed and considerably shortened the whole procedure. 500 grams of pig liver mince was stirred with 2.5 litres of redistilled acetone (precooled to -15°) for 15 minutes at -15°. The mixture was then filtered through a Buchner funnel at 2°, and the precipitate washed with more acetone (precooled to -15°). The residue was sucked dry in the funnel, immediately spread in a thin layer over paper, and dried for two hours at 2° with the aid of a fan. It was then dried in the same way at room temperature for a few hours, giving 167 g. of dry powder. This was stored at -15°.

Extraction of 25 g. lots of powder at room temperature (20-25°) was conducted by stirring with 250 ml. each of the solutions indicated in Table I-1. After the time indicated the mixture was centrifuged at 4000 g for 20 minutes at 2°, and the supernatant assayed for ATP:NMN adenylyltransferase activity and protein.

In the case of the water and 0.05 M-potassium chloride extractions, the residue, after centrifugation, was re-extracted for ten minutes with 0.1 M-disodium phosphate solution. Again after centrifugation as above, the supernatant was examined for enzyme and protein. The results are shown in Table I-1.

The yield of transferase, obtained by extraction of the acetone powder with 0.1 M-disodium phosphate, is about 70 units from 167 g. of powder, which is equivalent to 500 g. of fresh liver. As about 70 units of transferase is obtained from the 'nuclear' fraction of pig liver cells from 500 g. of liver by extraction with 0.2 M-disodium phosphate (Table I-6), there would seem to be little to choose between the two methods in obtaining an initial extract for large scale purification of the enzyme. However, the latter method, although rather tedious, can lead to higher specific activities with respect to the transferase in the initial extract, and so was finally adopted for the purification. The other extracting solutions indicated in Table I-1 were not very satisfactory for solubising the transferase. Although re-extraction of the 0.05 M-potassium chloride residue with 0.1 M-dipotassium phosphate did give a slight improvement in the specific activity of the transferase over that for initial extraction with 0.1 M phosphate, the yield was poor.

Table I-1

Extraction of ATP:NMN adenylyltransferase from pig liver acetone powder.

Extracting solution	0.1 M-Na_2HPO_4	0.1 M-Na_2HPO_4	0.05 M-KCl	0.05 M-KCl	water	0.01 M-Na_2CO_3	0.1 M-Na_2HPO_4 after 0.05 M-KCl	0.1 M-Na_2HPO_4 after water
Extraction time (minutes)	10	30	10	30	10	10	10	10
Transferase (units)	9.35	10.5	0	1.35	0	0	3.83	2.86
E_{280}/E_{260}	0.85	0.89	0.92	0.90	0.90	0.86	0.69	0.68
Protein (mg.)	6,400	10,500	5,500	7,650	5,640	6,400	1,700	2,090
Specific activity (units/mg.)	0.0015	0.0010	-	0.0002	-	-	0.0023	0.0014

(b) Extraction of ATP:NMN adenylyltransferase from the 'nuclear' fraction of pig liver.

The 'nuclear' fraction from pig liver cells was obtained by slicing up 100 g. of pig liver with scissors and blending in a Semak Vitamizer with 400 ml. of blending medium (see below) for three minutes. The homogenate was centrifuged for 20 minutes at 2° in an International refrigerated centrifuge (700 g). After the supernatant had been sucked off, the loosely packed pellet was mixed with more blending medium and centrifuged as above. This process was repeated once more. The final pellet was grey in colour and covered with a 'smear' of red cells. Microscopic examination showed it to consist largely of nuclei. For the small scale work described below, this pellet was resuspended in the blending medium.

The blending medium was made up by dissolving 342 g. of sucrose, 1 ml. of saturated calcium chloride and 5.3 ml. of triethanolamine in 3 litres of water. The hydrogen ion concentration was brought to pH 7.2 with approximately 3 ml. of 10 M-hydrochloric acid solution. After dilution to four litres, the solution was cooled to 2° before use.

ATP:NMN adenylyltransferase can be extracted from pig liver nuclei with 0.2 M-disodium phosphate (Atkinson, Jackson and Morton, 1961). Tables I-2 and I-3 show the results of a series of experiments in which the pig liver 'nuclear' fraction was extracted with 0.25 M-disodium phosphate and other solutions.

Table I-2

Extraction of ATP:NMN adenylyltransferase from the 'nuclear' fraction of pig liver. The freshly prepared 'nuclear' fraction, suspended in blending medium, was mixed with an equal volume (400 ml.) of the extracting solution to give the concentration indicated below. The mixtures were stirred for 30 minutes and centrifuged. The supernatants were assayed for transferase and for protein.

Extracting solution	1% detergent	M-NaCl	0.25M-Na_2HPO_4
Transferase (units)	18.1	16.4	18.6
E_{280}/E_{260}	0.69	0.65	0.65
Protein (mg.)	4800	2,800	1,085
Specific activity (units/mg. protein)	0.0038	0.0058	0.017

The data in Tables I-2 and I-3 are not strictly comparable as freshly prepared nuclei were used for the work described in Table I-2 and nuclei after storage frozen at -15° for that in Table I-3. However, freezing of the nuclei has little effect on the amount of extractable ATP:NMN adenylyltransferase, but does seem to lower the specific activity of the extract when 0.25M-disodium phosphate solution is used.

In addition to 0.25M-disodium phosphate solution, 3M-urea - 12.5% saturated ammonium sulphate (pH 7.0), 3M-urea (pH 7.0), 12.5% saturated ammonium sulphate (pH 7.0), 0.1M-pyridine - 0.02M-triethanolamine (pH 7.7), M-NaCl, 1% detergent and 25% saturated ammonium sulphate (pH 7.0), all extract significant amounts of the enzyme from the 'nuclear' fraction, in that order of decreasing effectiveness. Insignificant amounts of transferase are extracted by water, blending medium and 25% saturated ammonium sulphate - 0.25M disodium phosphate (Table I-3).

The case of 3M urea is an interesting one, in that it was the only solution tried which extracted ATP:NMN adenylyltransferase from the 'nuclear' fraction to give a higher specific activity than that of the 0.25M-disodium phosphate extract. Although it has been shown that partially purified transferase of specific activity 0.3 μmoles NAD synthesized per minute per mg. protein does not lose any activity on standing in 5M-urea for 48 hours at 2°, it was thought that the use of 3M-urea to extract the 'nuclear' fraction as a regular step in the purification of the enzyme was to be avoided. Alteration of proteins by urea solutions is well known (Stark, Stein and Moore, 1960).

Extraction of ATP:NMN adenylyltransferase from the 'nuclear' fraction by detergent appears to be approxiamtely proportional to the detergent concentration and to the amount of protein extracted. This situation holds until a concentration of 2.5% detergent is reached. At this concentration more protein is extracted, but transferase activity in the extract is not increased (Table I-4).

Table I-3

Extraction of ATP:NMN adenylyltransferase from the 'nuclear' fraction of pig liver. Conditions used were the same as described in Table I-2, except that the 'nuclear' fraction had been stored frozen for three days before use, and much smaller samples of the nuclear suspension were used (5 ml.).

Extracting solution	0.25M-Na_2HPO_4	3M urea - 12.5% saturated $(NH_4)_2SO_4$ (pH 7.0)	3M-urea (pH 7.0)	12.5 % saturated $(NH_4)_2SO_4$ (pH 7.0)	0.1M-pyridine-0.02M-triethanolamine (pH 7.7)	25% saturated $(NH_4)_2SO_4$ (pH 7.0)	25% saturated $(NH_4)_2SO_4$-0.25M-Na_2HPO_4	Blending medium (see text)	Water
Transferase in supernatant (units)	0.23	0.22	0.20	0.19	0.17	0.11	0.05	0.05	0.03
E_{280}/E_{260}	0.64	0.61	0.64	0.62	-	0.65	0.63	0.79	0.80
Protein in supernatant (mg.)	69.5	69.5	57.0	61.5	-	65.6	54.4	26.8	36.1
Specific activity (units/mg.)	0.0033	0.0031	0.0036	0.0031	-	0.0017	0.0009	0.0019	0.0009

However 1% detergent appears to extract most of the enzyme from the 'nuclear' fraction, and it is probable that at this concentration (1%) all the nuclear membranes have been broken up. At concentrations higher than 1%, the detergent is possibly solubizing protein by some other mechanism than the disruption of the nuclear membrane.

Table I-4

Extraction of ATP:NMN adenylyltransferase from the 'nuclear' fraction of pig liver by detergent. Conditions of the experiment were the same as described in Table I-3.

Detergent concentration % (v/v)	0	0.10	0.25	0.50	1.0	2.5
Enzyme extracted (units)	0.065	0.077	0.117	0.143	0.192	0.163
E_{280}/E_{260}	0.75	0.73	0.73	0.68	0.72	0.76
Protein (mg.)	27.2	38.4	44.8	54.4	72.0	123.0
Specific activity (units/mg.)	0.0024	0.0021	0.0026	0.0026	0.0027	0.0013

As indicated in Table I-4, the specific activity of transferase freed from the 'nuclear' fraction in the presence of up to 1% detergent is not very different from that which escapes from the nuclei suspended in blending medium in the absence of detergent. Since the latter is considered to be due to the mechanical disruption

of nuclear membranes in handling the materials (Hogeboom and Schneider, 1952), it is not surprising that similar specific activities are obtained in the presence of detergent if disruption of membranes is the only way in which detergent brings about liberation of transferase from the nucleus.

(c) Attempted partial purification of ATP:NMN adenylyltransferase from the M-NaCl extract of pig liver 'nuclear' fraction.

It has been shown by Hogeboom and Schneider (1952) that most of the ATP:NMN adenylyltransferase of the nucleus can be extracted into solution with a molar sodium chloride solution. They also demonstrated that if this solution is diluted six fold with water, about half the enzyme is co-precipitated with a fibrous precipitate containing nucleic acid. It was decided to investigate this phenomenon and attempt a purification of the transferase from a molar sodium chloride extract of nuclei.

Table I-2 shows the result of extracting the 'nuclear' fraction of pig liver with molar sodium chloride solution. When the extract was diluted with water (six fold), a stringy precipitate resulted. This was removed by centrifugation, leaving a supernatant with a specific activity of 0.014 with respect to the transferase. The supernatant contained 52% of the enzyme present in the original extract. An attempt was made to use this step in the attempted purification.

A 'nuclear' preparation from 800 g. of pig liver was obtained in the usual manner. This was suspended in 600 ml. of cold molar sodium chloride and stirred for 30 minutes at 2°. The suspension was centrifuged at 3,500g and the pellet discarded. The resulting supernatant was poured into 3.6 litres of cold water and stirred for 30 minutes at 2°. This was centrifuged as above. The supernatant was taken to pH 6.2 with cold 0.2M-acetic acid solution and the transferase adsorbed onto calcium phosphate gel with stirring. The amount of gel required was determined by enzyme assays. After collectinn of the gel by centrifugation, the enzyme was eluted with 0.5M-dipotassium phosphate solution, giving 372 ml. of eluate. This was subjected to ammonium sulphate fractionation, the precipitates obtained at 0-25, 25-35 and 35-45% saturation of ammonium sulphate being collected by centrifugation and dissolved in cold water. All three precipitates were white in colour, the 45% supernatant being red. Table I-5 shows the course of the purification.

This method of purification of ATP:NMN adenylyltransferase was abandoned because of the large volumes to be handled at the stage of dilution of the molar sodium chloride extract. Overall purification was very low - only about four fold from the original extract.

(d) Partial purification of ATP:NMN adenylyltransferase from a phosphate extract of the pig liver 'nuclear' fraction.

The 0.2M-disodium phosphate extract of the pig liver 'nuclear' fraction was obtained essentially as described by Atkinson, Jackson and Morton (1961). Further purification was carried out by

Table I-5

Partial purification of ATP:NMN adenylyltransferase from M NaCl extracts of pig liver 'nuclear' fraction.

	Protein (mg.)	E_{280}/E_{260}	Transferase (units)	Specific activity	Volume (ml.)
M-NaCl extract	6,180	0.64	80	0.013	840
Gel eluate	1,250	0.69	25.2	0.022	372
Ammonium sulphate fractionation					
0-25% Satn.	-	-	11.3	-	125
25-35% Satn.	204	0.87	9.4	0.046	60
35-45% Satn.	82	0.55	4.2	0.051	25

adsorbing the enzyme in the phosphate extract onto calcium phosphate gel which was formed by adding calcium chloride solution to the extract. It was then eluted from the gel and fractionated with ammonium sulphate.

Livers were removed at the slaughterhouse, packed in ice and used within an hour. 100 gram lots of fresh pig liver were sliced into small pieces and each lot homogenized in 400 ml. of 0.25M-sucrose - 0.003M-calcium chloride solution with a Waring blender at 2°. The blended material was filtered through a cheese cloth. When 4 litres of homogenate had been obtained it was centrifuged at 1000 g for 20 minutes at 2°. The loosely packed pellet was resuspended in the above medium to 4 litres and centrifuged again.

The resulting 'nuclear' fraction was suspended in 0.25M-sucrose - 0.015M-NaCl - 0.01M-disodium phosphate to a final volume of 3.6 litres and stirred for 30 minutes at room temperature (20-25°). After centrifugation at 1000g for 20 minutes (2°), the pellet so obtained was extracted with 0.2M-disodium phosphate solution (final volume 1.2 litres). After three to five minutes of vigorous stirring the mixture becomes extremely viscous as the intact nuclei break up, liberating material into solution. The mixture was stirred a further 60 minutes at room temperature (during this time the viscosity decreased), and centrifuged at 3500g for 60 minutes at 2°. The supernatant was stored frozen at -15°. 1.6 kilograms of pig liver could be handled in this way each day. Table I-6 shows the recovery of transferase at each stage in the preparation of a 'nuclear' extract.

The 0.2M-disodium phosphate 'nuclear' extract from approximately 13Kg. of pig liver was thawed and centrifuged at 1000g. The supernatant after standing overnight at 2° was poured off from a small amount of precipitated sodium phosphate. This gave a solution of total volume 16.4 litres with a pH of 8.5. Pilot experiments showed that the enzyme could be adsorbed onto calcium phosphate gel without previous lowering of the pH of the extract with dilute acetic acid, and that 0.2M-calcium chloride solution added to the extent of 20 ml. per 100 ml. of phosphate extract was sufficient to generate enough gel for total adsorption of the enzyme.

Table I-6

Recoveries of transferase in the preparation of a 'nuclear' extract from 800 g. of pig liver. Assays for transferase activity were conducted in the presence of 0.2M-nicotinamide, to inhibit NAD nucleosidase present in the initial homogenate.

Stage	Volume (ml.)	Transferase (units)
Sliced, blended and filtered	4,000	266
Centrifuged, resuspended in 0.25M-sucrose - 0.003M-$CaCl_2$	4,000	199
Centrifuged, suspended and stirred in 0.25M-sucrose - 0.015M-NaCl - 0.01M-Na_2HPO_4	3,600	218
Centrifuged, suspended and stirred in 0.2M-Na_2HPO_4	1,200	98.5
Centrifuged, final extract	730	114

To the phosphate extract, 3.3 litres of 0.2M-calcium chloride solution was added with constant stirring at 2° over a period of 30 minutes. The pH fell to 7.4. The whole batch was then centrifuged at 2,000g for 20 minutes at 2° and the supernatant discarded. The gel was suspended in 3.25 litres of 0.5M-sodium chloride - 0.02M-sodium phosphate, pH 6.4 and stirred at 2° for 30 minutes. The mixture was then centrifuged as above and the supernatant discarded. About 1.8 litres of elution fluid (0.21M-ammonium sulphate - 0.08M-disodium phosphate - 0.5mM-EDTA) was added to the gel and the mixture stirred for one hour at 2°. It was then centrifuged as before, and the

supernatant (first eluate) put aside. This procedure was repeated until four eluates were obtained. The stirring of the gel with elution fluid was carried out at 2°, and for times which varied from 5.5 hours to 13 hours. The second, third and fourth eluates were combined and fractionated with ammonium sulphate. The precipitate obtained at 28% saturation was discarded. At 45% saturation the precipitate was collected by centrifugation and dissolved in the minimum amount of cold water. This final solution keeps well for several months frozen at -15°. The overal yield from the 'nuclear' phosphate extract was 30% with a 57.5 fold purification. The enzyme had a specific activity of 0.54 μmoles NAD synthesized per minute per mg. protein. Table I-7 shows the details of this purification.

Table I-7

Partial purification of ATP:NMN adenylyltransferase from pig liver nuclei.

	Volume (ml.)	Units of enzyme	E_{280}/E_{260}	Protein (mg.)	Specific activity	Purification factor
Nuclear extract	16,400	1,000	0.695	106,000	0.0094	1
First gel eluate	1,750	74	0.98	1,770	0.042	4.5
Second gel eluate	1,900	198	0.96	1,750	0.12	12.8
Third gel eluate	1,100	101	0.97	1,144	0.088	9.4
Fourth gel eluate	1,500	73	1.00	885	0.083	8.8
Second, third and fourth eluates combined	4,500	372	0.97	3,779	0.10	10.5
28-45% Satd. $(NH_4)_2SO_4$ fraction	57	302	1.3	564	0.54	57.5

Another batch of 17.5 litres of 'nuclear' extract from approximately 14 kg. of pig liver was partially purified using the same procedure as above except that five eluates were obtained from the gel, and that eluates 2, 3, 4 and 5 were combined for fractionation.with ammonium sulphate. This gave a 41% overall yield with a 77.5 fold purification from the 'nuclear' extract. ATP:NMN adenylyltransferase with a specific activity of 0.52 μmoles NAD synthesized per minute per mg. of protein was obtained as indicated in Table I-8.

Table I-8

Partial purification of ATP:NMN adenylyltransferase from pig liver nuclei.

	Volume (ml.)	Units of enzyme	E_{280}/E_{260}	Protein (mg.)	Specific activity	Purification factor
Nuclear extract	17,500	730	0.74	108,400	0.0067	1
First gel eluate	1,360	22	1.15	1,960	0.011	1.6
Second gel eluate	1,880	175	1.08	2,400	0.073	10.9
Third gel eluate	1,260	114	1.02	1,220	0.093	13.9
Fourth gel eluate	1,380	89.5	0.97	883	0.10	15.0
Fifth gel eluate	1,420	53	0.91	795	0.067	10.0
Second - fifth eluates combined	5,940	432	1.0	5,298	0.082	12.2
28-45% $(NH_4)_2SO_4$	37	300	1.3	574	0.52	77.5

(e) Chromatographic behaviour of ATP:NMN adenylyltransferase preparations.

DEAE cellulose

In general, chromatography of the transferase preparations on DEAE cellulose can be carried out in two ways. If the preparation contains relatively large amounts of nucleic acid, much of this material can be removed by chromatography at pH 6.5, and a small but useful amount of contaminating protein removed as well. Under these conditions the transferase emerges from a column of DEAE cellulose at the 'break-through' point. The other method is to adsorb the transferase onto a DEAE cellulose column at pH 7.5 and elute by applying a salt gradient. The second method gave varying results with different transferase preparations and generally is less useful than the first. A description of each method is given in (i) and (ii) below.

(i) DEAE cellulose chromatography at pH 6.5. A column of DEAE cellulose was washed with 0.2M-K_2HPO_4, water and finally with 10 litres of 0.025M-potassium phosphate, pH 6.5. The final dimensions of the columnwere 12.1 cm. long x 2.5 cm. diameter. To 40 ml. of a solution of ATP:NMN adenylyltransferase obtained by a method similar to that described in Table I-7, 5 ml. of 0.2M-potassium phosphate, pH 6.5, was added. The preparation had a specific activity of 0.56 μmoles NAD synthesized per minute per mg.protein and contained relatively large amounts of nucleic acid (E_{280}/E_{260}= 0.92). This solution (at 2°) was added to the top of the cellulose column and washed through with 0.025M-potassium phosphate, pH 6.5. The eluate was collected in fractions and each fraction tested for its protein and transferase content. Table I-9 shows the results of the experiment.

Table I-9

Chromatography of ATP:NMN adenylyltransferase on DEAE cellulose at pH 6.5.

Fraction number	Volume (ml.)	Transferase (units)	E_{280}/E_{260}	Protein (mg.)	Specific activity
1	25	0	-	-	-
2	13	0	-	-	-
3	6.5	6.3	1.17	6.5	0.97
4	6.5	10.0	1.54	11.4	0.88
5	6.5	15.8	1.50	15.6	1.01
6	6.5	15.9	1.47	16.9	0.94
7	6.5	14.7	1.48	16.9	0.87
8	6.5	12.0	1.48	15.0	0.80
9	6.5	7.6	1.43	17.1	0.45
10	10	1.6	1.22	2.0	0.8
11	8	0	-	0	-

A recovery of 68% of the transferase was obtained. Better recoveries can be attained using smaller amounts of DEAE cellulose (in relation to the amount of protein added), but in these cases the purification achieved is much less than that indicated in Table I-9.

(ii) DEAE cellulose chromatography at pH 7.5. A column of DEAE cellulose was washed with six litres of 0.01M-sodium phosphate pH 7.5. The final dimensions of the column were 1.8 cm. diameter x 11 cm. long. 9 millilitres of an ATP:NMN adenylyltransferase

preparation (Table I-8) containing 89 mg. of protein and 48 units of ATP:NMN adenylyltransferase was dialysed against two changes (2 litres each) of 0.01M-sodium phosphate, pH 7.5, overnight at 2°. A precipitate developed after several hours. Next day the dialysed material was centrifuged at 10000g for 25 minutes.

Nine ml. of the supernatant was applied to the DEAE cellulose column. After adsorption of the transferase preparation, the column was washed with 35 ml. of 0.01M-sodium phosphate, pH 7.5, and then with approximately 60 ml. of 0.08M-NaCl - 0.01M-sodium phosphate, pH 7.5. Up to this stage no transferase activity had appeared in the eluate. Much of the transferase was eluted by means of a linear salt gradient from 0.08M-NaCl - 0.01M-sodium phosphate, pH 7.5 to 0.2M-NaCl - 0.01M sodium phosphate, pH 7.5. More enzyme was eluted by passing a solution of 1M-NaCl - 0.01M-sodium phosphate, pH 7.5 through the column. Fractions of 5 to 10 ml. were collected throughout and the whole operation was conducted at 2°. Each fraction was tested for ATP:NMN adenylyltransferase activity and the protein estimated by the method of Lowry et al. (1951).

Figure I-1 shows the results of the experiment in detail. A total of approximately 50% of the transferase was recovered from the anion exchange cellulose. Most of that recovered was eluted by a sodium chloride concentration of 0.08 to 0.12M (in the presence of 0.01M-sodium phosphate, pH 7.5) in two distinct peaks, and the remainder by 1M-sodium chloride - 0.01M-sodium phosphate, pH 7.5. The specific activity of the two peaks obtained at approximately

Fig. I-1

Chromatography of ATP:NMN adenylyltransferase on DEAE cellulose.

_____ units of transferase

\- - - - - - absorption at 280mμ

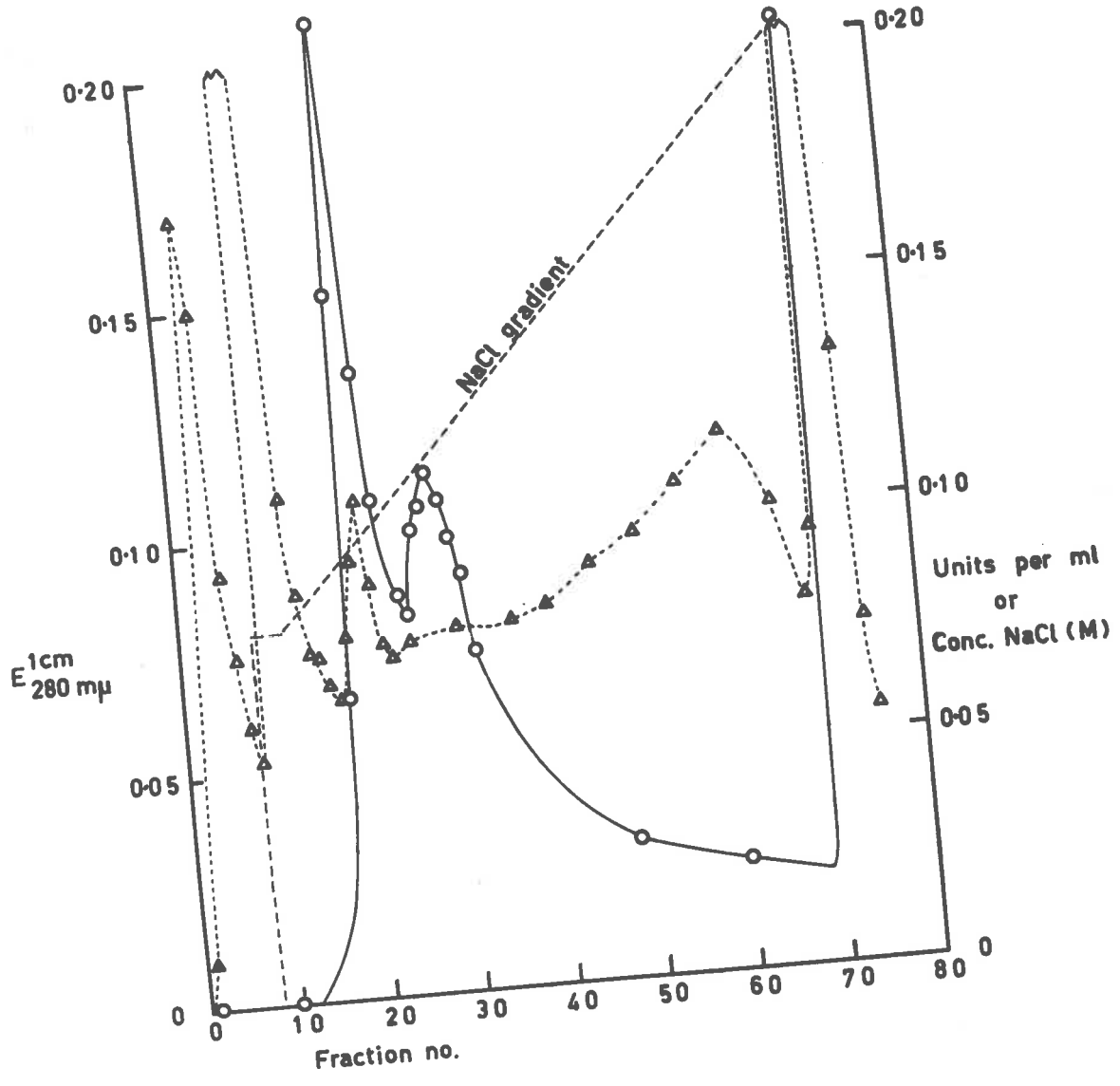

0·20
0·15
0·10
0·05
0
$E^{1cm}_{280\ m\mu}$
NaCl gradient
0·20
0·15
0·10
0·05
0
Units per ml
or
Conc. NaCl (M)
0
10
20
30
40
50
60
70
80
Fraction no.

Fig. I - 1

0.1M-sodium chloride is shown in figure I-2. The most active fraction had a specific activity of 2.33 μmoles NAD synthesized per minute per mg. of protein. This represents a purification of 4.4 fold over the material applied to the column. Although it is recognised that the two peaks obtained could be due to an uneven flow of solutions through the column, it is an interesting result when considered in the light of the experiments where transferase preparations were subjected to starch gel electrophoresis. Here at least two 'isoenzymes' of the transferase were apparent.

Chromatography of the transferase on DEAE cellulose at pH 7.5 under the above conditions was carried out a number of times and always resulted in the appearance of multiple peaks in the region of 0.1M-sodium chloride. From preparations of specific activity 0.25 to 0.5 μmoles NAD synthesized per minute per mg. protein, a purification of three to four fold only was achieved in the most active fractions. Use of this method in a routine purification of ATP:NMN adenylyltransferase does not seem warranted in view of the low yields of transferase with significantly increased specific activity.

(iii) Carboxymethyl cellulose. ATP:NMN adenylyltransferase preparations were chromatographed on the cation-exchanger, CM cellulose, at pH 6.5 and 5.0, with little improvement in specific activity.

Dialysis of a transferase solution containing 8.45 units of specific activity 1.19 in 4.0 ml. against 0.1M-sodium acetate (pH 5.0) - 0.1 mM-EDTA for six hours at 2°, gave a preparation containing 2.1 units

Fig. I-2

Chromatography of ATP:NMN adenylyltransferase on DEAE cellulose.

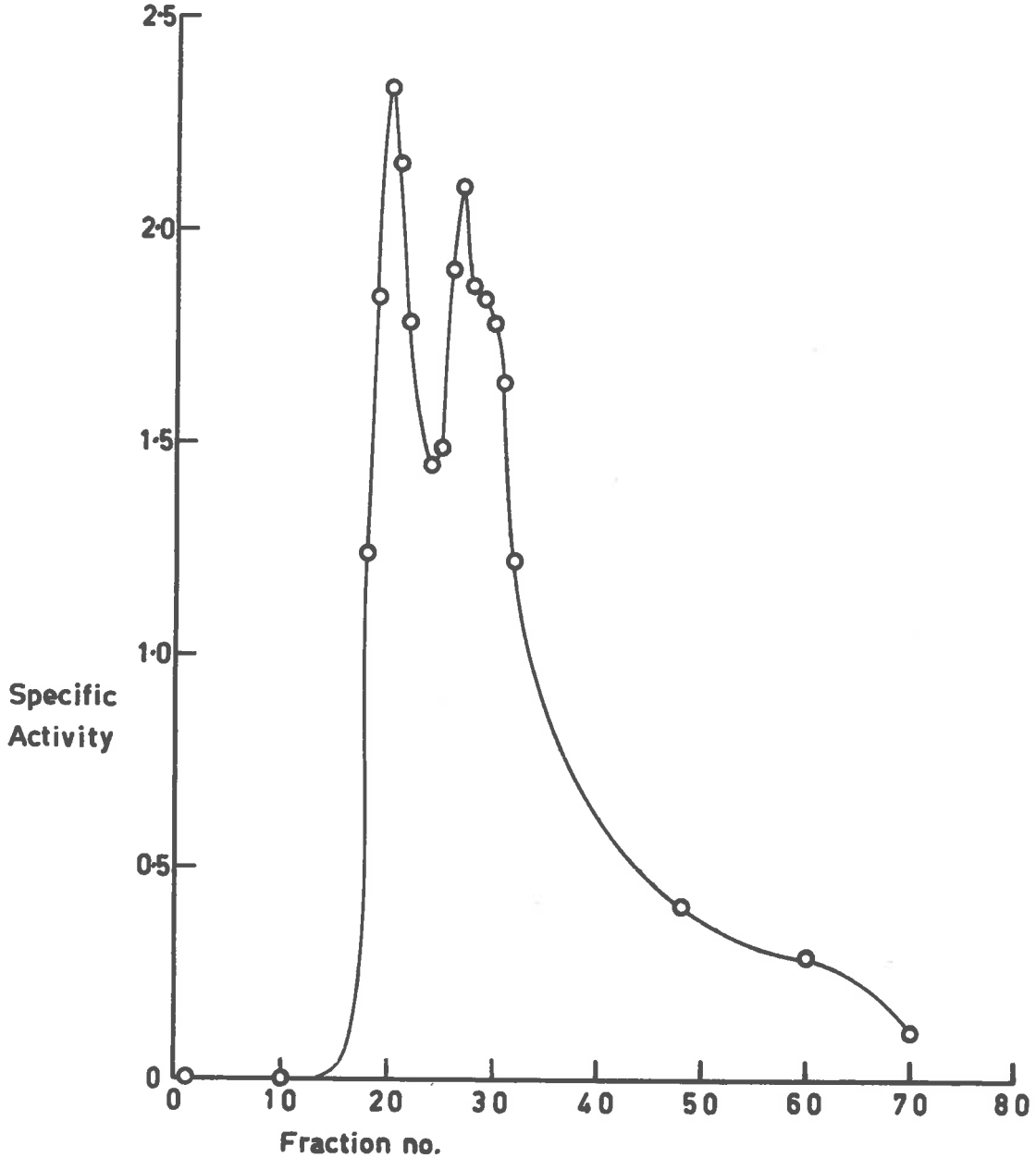

2·5
2·0
1·5
1·0
0·5
0
Specific
Activity
0
10
20
30
40
50
60
70
80
Fraction no.

Fig. I - 2

of specific activity 0.93 in 4.4 ml., after centrifugation to remove a precipitate. A recovery of only 25% of the transferase was obtained after dialysis at pH 5.0. This solution was applied to a column of CM cellulose 2.5 cm. diameter x 3 cm. long, which had previously been washed with 0.5M-sodium hydroxide, 0.5M-sodium chloride and finally with 0.1M-sodium acetate, pH 5.0 - 0.1mM-EDTA, until an eluate of pH 5.0 was obtained. The transferase was found to be adsorbed to the cation exchanger, and only about 25% of this could be eluted with 1M-sodium chloride - 0.1mM-EDTA. The specific activity of the eluted enzyme was approximately 1.0.

Another column was set up with similar dimensions and washed successively with 0.5M-sodium hydroxide, 0.5M-sodium chloride, 0.01M-sodium chloride, 0.1M-sodium acetate, pH 4.55 and finally with 0.01M-sodium chloride. An enzyme solution containing 14.2 units of specific activity 0.34 in 12.5 ml. (pH 6.5) was added to this column. Most of the transferase was eluted at the 'break-through' point and more appeared after elution with 0.1M-sodium chloride and M-sodium chloride. A total of 80% of the transferase was recovered, with specific activities ranging from 0.2 to 0.7 for the various fractions collected. In another experiment carried out under the same conditions, 54% of the added transferase preparation was eluted at the 'break-through'. In this case 49 units of transferase (specific activity 1.03) in 5.3 ml. was applied to the cation exchanger, and 26.4 units recovered with a specific activity of 1.05 to 1.48.

(iv) Amberlite CG-50. In general the behaviour of ATP:NMN adenylyltransferase on the cation exchange resin Amberlite CG-50 is consistent with the enzyme molecule having an iso-ionic point in the neutral pH region. At pH 7.0, (0.05M-sodium phosphate buffer) about 50% of the transferase is eluted from a column of the resin at the 'break-through' point. All the transferase is adsorbed by the resin at pH 5.8 (0.02M-sodium phosphate buffer).

A column of Amberlite CG-50 of dimensions 1.4 cm. diameter x 13.3 cm. long was equilibrated with 0.05M-sodium phosphate, pH 7.0. In preparation for chromatography, a transferase sample was dialysed against 0.05M-sodium phosphate, pH 7.0 for four hours at 2°. This gave 25 ml. of a solution containing 43 units of transferase with a specific activity of 0.28. The dialysed material was added to the resin, and the column eluted successively with 0.05M-sodium phosphate, pH 7.0, 0.2M-sodium phosphate, pH 7.0 and finally with 0.2M-disodium phosphate solution. The eluates were collected as 8 ml. fractions, and assayed for their protein and transferase content. The elution pattern is shown in figure I-3. Recovery of transferase from the column was about 80%, and 90% of the protein was accounted for. Approximately 50% of the transferase recovered appeared at the 'break-through' with a relatively low specific activity (0.18), and the remainder was eluted by 0.2M-sodium phosphate, pH 7.0 with an increased specific activity (approximately 0.6).

Dialysis of 10 ml. of a solution containing 26 units of transferase (specific activity 0.3) against four litres of 0.02M-sodium

Fig. I-3

Chromatography of ATP:NMN adenylyltransferase on Amberlite CG-50

- - - - - - protein

———— units of transferase

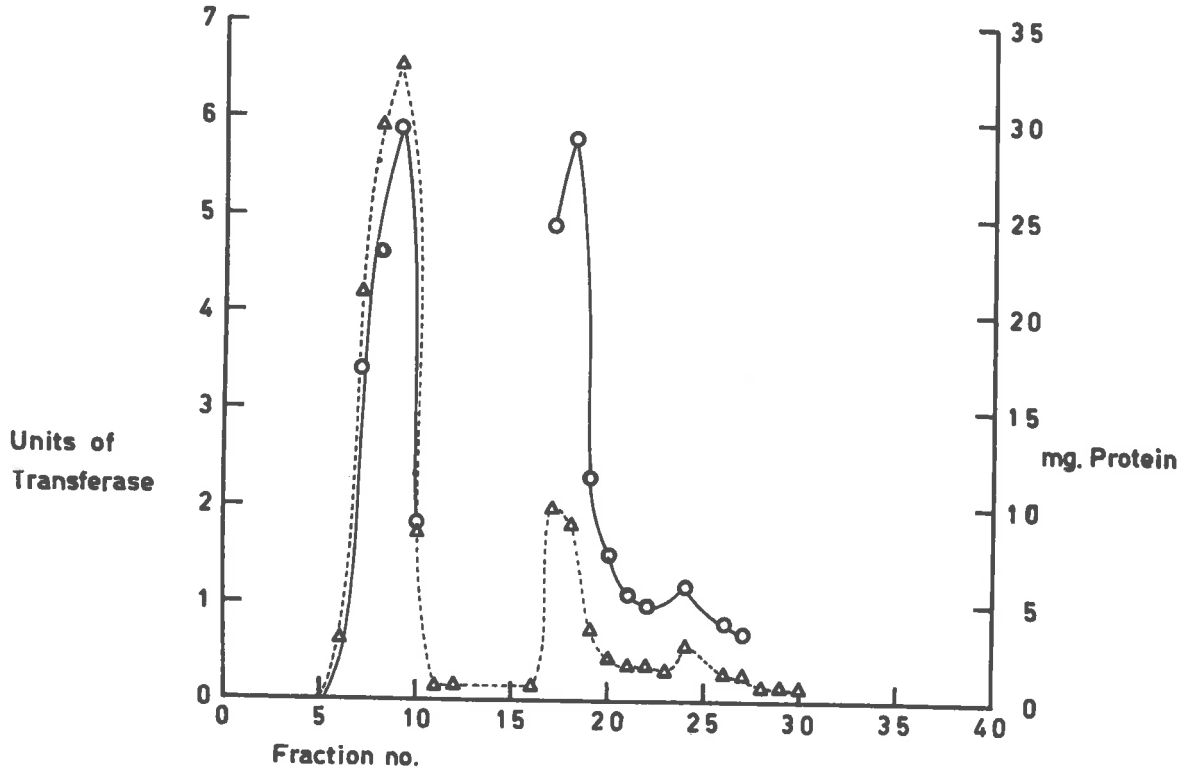

Units of
Transferase
mg. Protein
Fraction no.
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
0
5
10
15
20
25
30
35

Fig. I - 3

phosphate, pH 5.8 for 7 hours at 2° gave a precipitate. The supernatant, obtained by centrifugation, had 16.5 units of the enzyme with a specific activity of 0.26. This was found to be adsorbed to a column of the resin of the same dimensions as above, but previously washed with 0.02M-sodium phosphate, pH 5.8. Only some inactive protein was eluted with 0.02M-sodium phosphate, pH 5.8. The resin was then eluted with 0.04M-sodium phosphate, pH 6.5, 0.04M-sodium phosphate, pH 7.0 and finally with 0.2M-sodium phosphate, pH 7.0. Very little protein and no transferase was eluted with the first two solutions. Approximately 11% of the transferase was recovered by elution with 0.2M-sodium phosphate, pH 7.0, the most active fraction having a specific activity of 0.32.

Chromatography of the transferase preparations on Amberlite CG-50 in the presence of urea gives a small but useful increase in specific activity. Three ml. of a solution containing 9.2 units of transferase (specific activity 0.3), which had previously been dialysed versus 6.1M-urea - 0.146M-sodium phosphate, pH 6.15 without loss of activity or decrease in specific activity, was added to a column of Amberlite CG-50, 1.3 cm. diameter x 15.2 cm. long. This column had been washed extensively with 6.1M-urea - 0.146M-sodium phosphate, pH 6.15. Protein was washed through the column with the starting solution (urea-phosphate, pH 6.15). A total of 3.9 units of transferase was eluted with a specific activity ranging from 0.5 to 0.6. Thus 42.4% of the transferase activity and 21.5% of the protein was recovered. However, when the transferase was chromatographed on

this cation-exchanger in the presence of 5M-urea - 0.05M-sodium phosphate, pH 6.0, less than 10% of the enzyme was recovered.

(v) Calcium phosphate. A limited amount of work only has been carried out on the chromatographic behaviour of ATP:NMN adenylyltransferase on calcium phosphate preparations. The results obtained were very encouraging, especially as good recoveries were found. This method of purification deserves further attention. The calcium phosphate used was prepared by the method of Main, Wilkins and Cole (1959) by precipitation at pH 6.7 and boiling with saturated calcium hydroxide solution. Before use, a column of this material was washed with 200 ml. 0.1M-potassium phosphate, pH 6.6, followed by 2 litres of 0.01M-potassium phosphate, pH 6.6 - 0.01mM-cysteine.

To such a column of calcium phosphate (1.2 cm. diameter x 2.8 cm. long) 5 ml. of a transferase preparation containing 19.3 units of specific activity 0.98 (E_{280}/E_{260} = 1.37) in 0.01M-potassium phosphate, pH 6.6 was added. The column was immediately eluted with 0.35M-potassium phosphate, pH 6.6. The best fraction contained 18.2 units (94% recovery) of specific activity 1.57 (E_{280}/E_{260} = 1.46).

This fraction was fractionated with ammonium sulphate, and the material precipitating between 35 and 45% saturation was dissolved in 0.5 ml. of cold water to give a solution with 4.8 units of the transferase of specific activity 1.4 (E_{280}/E_{260} = 1.65). This was applied to another column of calcium phosphate, 1.2 cm. diameter x 2.9 cm. long. The column was eluted successively with 0.1M-potassium phosphate, pH 6.6, 0.2M-potassium phosphate, pH 6.6 and finally with 0.35M potassium phosphate, pH 6.6. Three ml. fractions were collected.

A total recovery of the transferase of 75% was achieved, most of this appearing on elution with the last mentioned solution. However, little further purification was obtained, the best fraction containing 2.3 units of specific activity 1.66 (E_{280}/E_{260} = 1.59).

(f) The effect of urea on ATP:NMN adenylyltransferase.

ATP:NMN adenylyltransferase preparations are remarkably stable in solutions containing up to 6M-urea. It is this property which allowed chromatography on Amberlite CG-50 to be conducted in the presence of urea, and which permitted the extraction of active transferase from pig liver nuclei by solutions containing urea.

Three experiments illustrate this stability towards urea. A transferase preparation containing 8.5 units of enzyme of specific activity 0.31 (E_{280}/E_{260} = 1.12) was dialysed against 5M-urea - 0.05M - sodium phosphate, pH 6.0 overnight at 2°. The dialysed solution was then kept at 2° for 48 hours. There was no loss in transferase activity. This solution was dialysed against 0.05M-sodium phosphate, pH 7.0 overnight at 2°. The resulting precipitate was removed by centrifugation. The supernatant was found to contain 80% of the original transferase with a specific activity of 0.78 (E_{280}/E_{260} = 1.07). A similar behaviour was found after dialysing 40 units (specific activity 0.3) of transferase against 5M-urea - 0.04M-sodium phosphate, pH 5.8, and keeping the solution for 48 hours at 2°. In this case, subsequent dialysis of the solution against 0.05M-sodium phosphate, pH 7.0, gave a 96% recovery of the transferase

in the supernatant with a specific activity of 0.8.

An increase in specific activity of the enzyme was not attained during a shorter treatment of this kind. In another experiment 3 ml. of a 0.025M-potassium phosphate solution, pH 6.5, containing 7.8 units of transferase (specific activity 0.3) was made up to 5M-urea by the addition of solid urea and kept for 30 minutes at 2°. This was dialysed against water overnight at 2°. After centrifugation to remove a precipitate, the supernatant (4.4 ml.) was found to have 6.46 units of transferase of specific activity 0.28 (83% recovery).

Tables I-7 and I-8 above show a recovery of 81 and 69.5% respectively of transferase in the precipitate obtained at 28 to 45% saturation during ammonium sulphate fractionation. Frequently lower recoveries are obtained, and in at least one case, to be described, the presence of urea has resulted in a higher recovery in this fraction during treatment with ammonium sulphate.

Two solutions of transferase in 30 ml. of 0.5M-dipotassium phosphate, each containing 24.8 units of enzyme of specific activity 0.07, were fractionated with ammonium sulphate at 2°. The precipitates obtained at 25 and 45% saturation with ammonium sulphate were dissolved in ice-cold water and assayed for their transferase and protein content. The 25 to 45% fraction, obtained in the presence of 2M-urea, contained 17 units with a specific activity of 0.61, representing a recovery of 68.5%. That obtained in the absence of urea yielded 7.1 units with a specific activity of 0.7 (30% recovery). In the latter case, more transferase of low specific activity was obtained in the 0-25%

saturation fraction than was the case in the presence of urea. This may indicate that the urea is acting by preventing to some extent the interaction of the enzyme with other proteins.

(g) Acetone fractionation of ATP:NMN adenylyltransferase preparations.

ATP:NMN adenylyltransferase with a specific activity of approximately 0.5 μmoles NAD synthesized per minute per mg. protein can be readily prepared by the procedures outlined in Tables I-7 and I-8. The purity of these preparations can be increased to a specific activity of about 1.0 to 2.3 by some of the techniques described above. Fractionation with acetone also gives a useful purification of the transferase, leading to preparations capable of synthesizing 2 to 3 μmoles NAD per minute per mg. protein. However, in general, this method gives only relatively low yields of enzyme of this purity. The course of fractionation of the transferase with acetone is shown in Tables I-10 and I-11.

The starting material for the fractionation summarised in Table I-10 was obtained by dialysing a transferase preparation against 0.025M-potassium phosphate, 0.01mM-EDTA, pH 6.5, for 16 hours at 2°. The mixture was then centrifuged and the supernatant cooled at 0° in an alcohol bath. Acetone (precooled to -15°) was added slowly until it made up 15% of the solution; the temperature of the mixture was allowed to drop to -10° during addition. The slight precipitate was isolated by centrifugation at 20000g at -15°. In this way, fractions were collected at 30 and 50% acetone.

Table I-10

Acetone fractionation of ATP:NMN adenylyltransferase.

Starting material: 125 units transferase
1.03 specific activity
105 ml. 0.025M-potassium phosphate
0.01mM-EDTA (pH 6,5)

Precipitated by:	Soluble in 0.025M phosphate		Soluble in 0.1M phosphate	
% acetone	units	specific activity	units	specific activity
15	3.35	2.2	9.0	2.68
30	3.32	1.23	2.64	1.90
50	15.4	-	10.8	-
(supernatant)	0.5	-	-	-

The precipitates were suspended in 0.025M-potassium phosphate, pH 6.5. These suspensions and the supernatant obtained after collecting the 50% acetone precipitate were dialysed overnight against 0.025M-potassium phosphate, pH 6.5, at 2°. As only a portion of each precipitate appeared to be soluble in this buffer, the next day all residues were dissolved in 0.1M-disodium phosphate, and the various solutions obtained were assayed for transferase and protein. The purest fractions were obtained at 15% acetone; however, most of the transferase recovered was precipitated at 50% acetone (Table I-10). An overall recovery of 36% of transferase was found.

Table I-11

Acetone fractionation of ATP:NMN adenylyltransferase.

Starting material: 105 units transferase
0.47 specific activity
30 ml. 0.025M-potassium phosphate,
0.01mM-EDTA (pH 6.5)

% acetone	units	specific activity
16.5	10.1	3.3
33	8	0.7
(supernatant)	78	-

The fractionation summarized in Table I-11 was conducted under the conditions described in Table I-10. In this case the starting material was the supernatant obtained after dialysing an enzyme preparation of specific activity 0.61 against 0.025M-potassium phosphate 0.01mM-EDTA,pH 6.5, for 16 hours at 2°. Only two fractions were collected (at 16.5 and 33% acetone). The precipitates were suspended in 0.025M-potassium phosphate, pH 6.5 and dialysed against this solution overnight at 2°. Both dissolved completely in this buffer. The supernatant, containing 33% acetone, was dialysed against this buffer to rid it of acetone. Again, the best fraction was that obtained at 16.5% acetone. This represented less than 10% of the transferase recovered. A total recovery of 91% was obtained, most of the transferase being soluble in 33% acetone solutions. Further experiments on

acetone fractionation of ATP:NMN adenylyltransferase preparations are described in the following section.

(h) Starch gel electrophoresis of ATP:NMN adenylyltransferase preparations.

Continuous buffer systems.

ATP:NMN adenylyltransferase of specific activity 0.82 was obtained by the procedures outlined in Tables I-7 and I-8 followed by chromatography on DEAE-cellulose at pH 6.5. A sample of this pig liver enzyme (0.075 ml. containing 0.7 units) was subjected to starch gel electrophoresis in 0.025M-boric acid - 0.01M-sodium hydroxide, pH 8.4, according to the method of Smithies (1959). A voltage of 270 volts (10V/cm.) was applied across the gel for a period of 6.5 hours at 2°. At the end of the run, the starch block was sliced into two equal parts in the usual manner. One half was stained for protein with amido black solution. Two protein components were observed at a distance of 1.4 and 1.7 inches on the anode side of the origin. There was a large concentration of adsorbed protein at the origin, and a pronounced streaking of protein from the origin towards the anode was evident. The other half of the gel was sliced into 0.25 inch sections moving down the block from the origin towards the anode. The sections were frozen overnight at -15°, thawed the next day and each placed on the 'mushroomed' end of a glass rod which rested in a centrifuge tube. The 'mushroom' was held at a distance of approximately one inch from the bottom of each tube by a constriction in the latter. These tubes containing the

gel sections were centrifuged at 300g for 30 minutes to express the solution from the spongy starch. The liquid collected in the bottom of each tube (approximately 0.2 ml.) was assayed for its transferase activity by incubation with 0.8 ml. of a solution containing one μmole of NMN, 4 μmoles of ATP, 16 μmoles of $MgCl_2$ and 200 μmoles of glycylglycine, pH 7.6. The incubation was carried out at 37° for 45 minutes. Only a trace of activity was found in the liquid from the section taken from the origin, and no detectable NAD synthesis was apparent in the solutions from the other sections. In view of the following experiment, it seems that the transferase is rather firmly attached to the starch under the above conditions, perhaps due to freezing of the gel before expression of the solution it contains.

Starch gel electrophoresis of the same enzyme preparation was repeated under the conditions stated above. For a period of 13.75 hours, 270 volts was applied across the gel. The gel was split horizontally as before. Staining with amido black solution showed that the two protein bands observed above had moved a distance of 4.1 and 4.2 inches towards the anode from the origin. Again there was much protein adsorbed at the origin, and heavy 'streaking' of protein away from the origin towards the anode was apparent. As above, the other half of the gel was cut into 0.25 inch sections down the starch gel block from the origin toward the anode. Each section was placed in 0.5 ml. of water and macerated. To each of the resultant suspensions, 0.4 ml. of a solution containing one μmole

of NMN, 4 μmoles of ATP, 16 μmoles of $MgCl_2$ and 200 μmoles of glycylglycine, pH 7.6 was added, and the mixture incubated at 37°. The reaction was stopped with trichloroacetic acid in the usual way. From the amount of NAD so formed, the relative activity of transferase in each section was ascertained. Figure I-4 (a) shows the distribution of transferase activity in relation to the observed protein components. ATP:NMN adenylyltransferase activity was observed to cover a large area of the block, and could not be allocated to any distinct protein component, except perhaps a portion of it to that protein diffusing out from the origin towards the anode. At a specific activity of 0.82, the transferase is evidently far from pure, or is modified by the conditions of electrophoresis.

The techniques of Smithies (1959) were used to conduct two more investigations into the electrophoretic behaviour of transferase preparations on starch gel. Here the starch gel was made up in 0.01M-sodium phosphate buffers, pH 7.5 and 6.8. Each buffer had a specific conductance of 6.6×10^{-4} $ohm^{-1}cm.^{-1}$, which was found suitable for electrophoresis under these conditions. A transferase preparation obtained as described in Table I-7, was dialysed against these two buffer solutions, giving enzyme of specific activity 0.54. 0.4 units of enzyme at pH 6.8 and 7.5 were applied to the gels at the same pH, and 150V was applied across each gel (27 cm.) for six hours at 2°. The procedures outlined above were employed to detect protein and transferase activity. The distribution of transferase and protein is shown in Figures I-4 (b) and I-4 (c). At pH 6.8 the transferase

Fig. I-4

Starch gel electrophoresis of ATP:NMN adenylyltransferase preparations

_____ transferase

//// protein

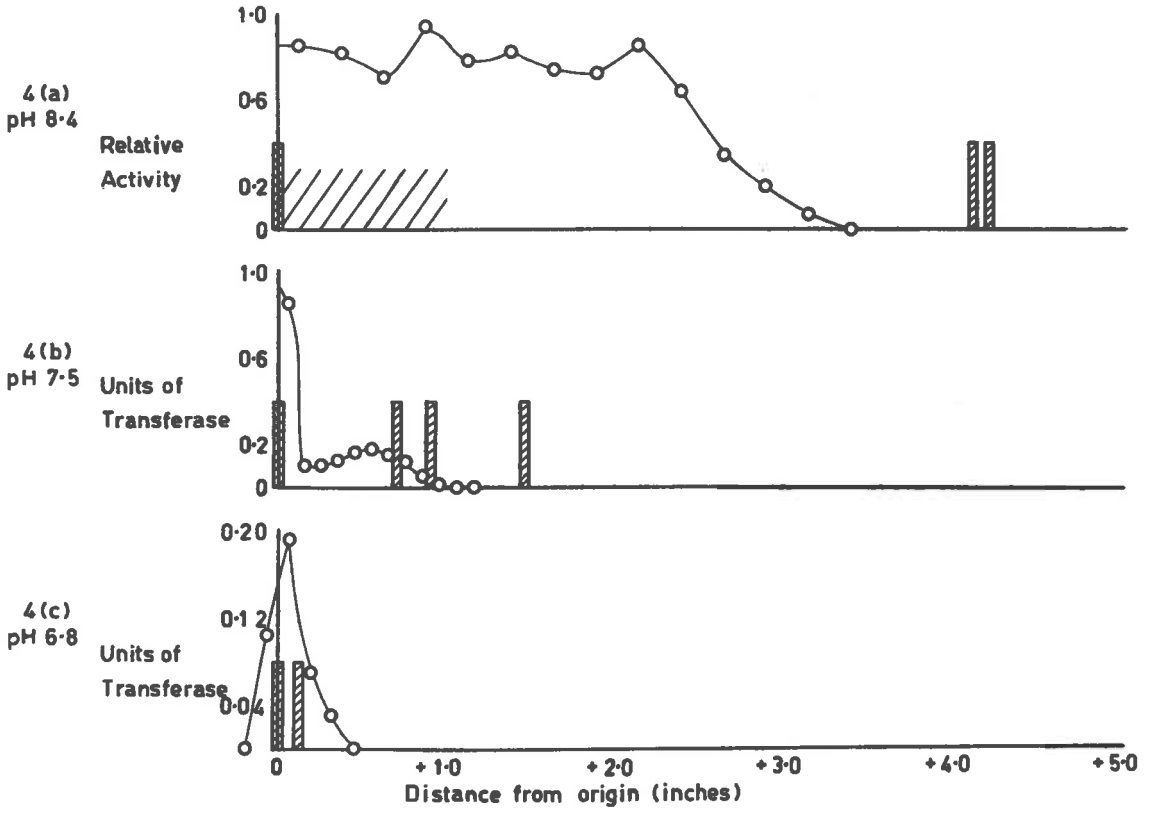

4(a)
pH 8·4
Relative
Activity
1·0
0·6
0·2
0
4(b)
pH 7·5
Units of
Transferase
1·0
0·6
0·2
0
4(c)
pH 6·8
Units of
Transferase
0·20
0·12
0·04
0
+1·0
+2·0
+3·0
+4·0
+5·0
Distance from origin (inches)

Fig. 1-4

appears to have little net negative charge as most of the activity has remained near the origin (figure 4 (c)). At pH 7.5 (figure I-4 (b)) a significant portion has migrated towards the anode, whereas at pH 8.4 (figure I-4 (a)) most of the transferase has moved away from the point of application. The preparation subjected to electrophoresis at pH 8.4, which had been passed through a DEAE cellulose column at pH 6.5, is devoid of the third protein component seen at electrophoresis at pH 7.5, where the transferase applied was not given this additional treatment. This indicates that it is the removal of this component that results in the rise in specific activity from 0.5 to 0.8.

Discontinuous buffer system.

The following experiments, describing the behaviour of ATP:NMN adenylyltransferase preparations on starch gel electrophoresis, were carried out using the discontinuous buffer system of Poulik (1957). The transferase was detected by the coupled enzymic test, details of which are given in the methods section above.

(i) An enzyme preparation originally of specific activity 0.52 (obtained as in Table I-8), after having been kept frozen at -15° for 6 months was found to have a specific activity of 0.31. After dialysis against 0.005M-sodium pyrophosphate-HCl, pH 8.0 at 2°, the specific activity was raised to 0.36. Two ml. of this solution, containing 7.1 units was added to 2 ml. of acetone at -15°. A heavy precipitate resulted, which was collected by centrifugation at 20000g for ten minutes at 2°. The precipitate was dissolved in

0.005M-sodium pyrophosphate-HCl, pH 8.0, and dialysed against this buffer overnight at 2°. 1.2 millilitres of a yellow solution containing the transferase was obtained. Of this 0.7 ml. was centrifuged at 259000g for 30 minutes at 4° (Model E Spinco). A hard yellow pellet was found at the bottom of the cell at the end of the run. 0.5 millilitres of the supernatant was withdrawn with a syringe, yielding a colourless ATP:NMN adenylyltransferase preparation of specific activity 0.62 (5.15 units per ml., E_{280}/E_{260} = 1.35).

Two slots were used for starch gel electrophoresis. The right hand slot contained the preparation before centrifugation on the Spinco, and the left hand slot, the supernatant after this treatment. As can be seen in figure I-5, centrifugation at 259000g removes high molecular weight material (yellow), which becomes adsorbed to, and streaks out from, the origin on starch gel electrophoresis. Some ATP: NMN adenylyltransferase is associated with the major protein band which moves with a mobility of slightly more than half that of the 'borate front'. This electrophoresis was carried out by applying a potential of 105 volts across the gel for 5 hours. A high voltage like this appears to distort the pattern somewhat. The left side of figure 5 shows the protein pattern, while regions of transferase activity are depicted at the right.

(ii) 47.2 Units of transferase of specific activity 0.26 was freshly prepared from pig liver nuclei as described in Table I-8. After dialysis against 0.005M-sodium pyrophosphate - HCl, pH 8.0 at 2° overnight, it was subjected to an acetone fractionation. At 40%

Fig. I-5

Starch gel electrophoresis of ATP:NMN adenylyltransferase preparations.

The origin in situated at the top of the page.

(see text)

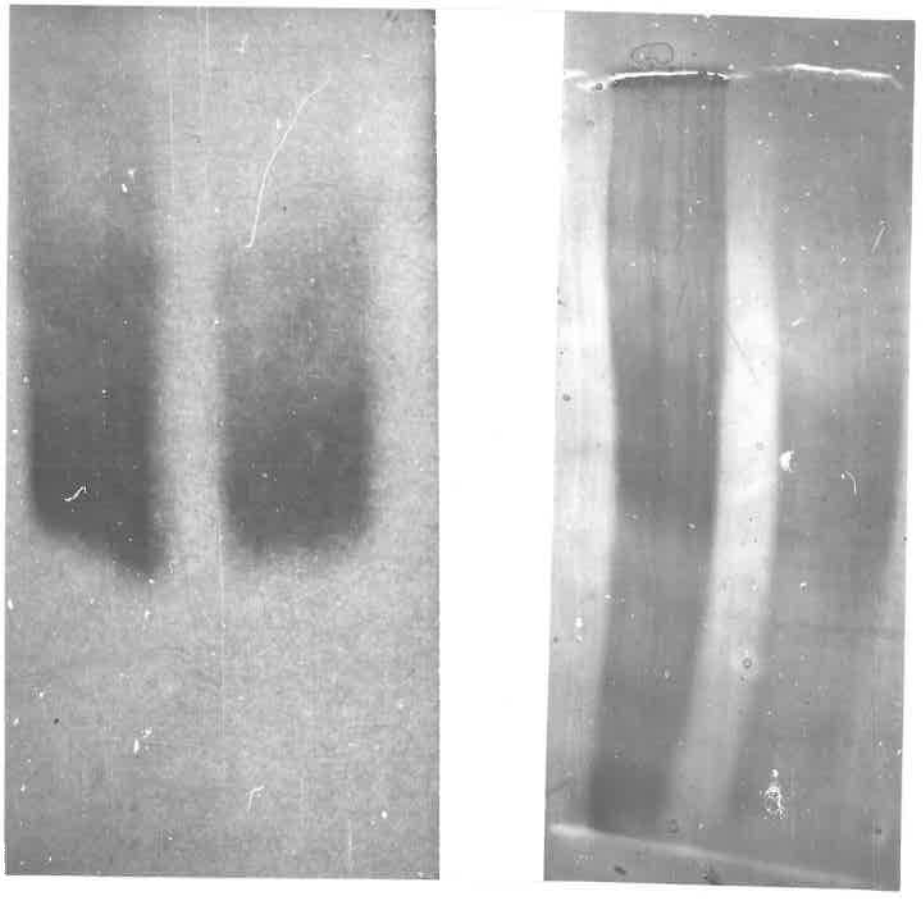


Fig. I-5

acetone (-15°) fine crystals appeared. They were collected by centrifugation (20,000 x g for 10 minutes), but little transferase activity was associated with them. At 60% acetone a heavy precipitate resulted. This was removed by centrifugation as before and suspended in 0.005M-sodium pyrophosphate-HCl, pH 8.0. After dialysis against this buffer overnight, the small amount of insoluble material was removed by centrifugation. The supernatant contained 31 units of transferase of specific activity 0.24 (66% yield).

To improve the specific activity, this material was subjected to an ammonium sulphate fractionation. Fractions were collected at (1) 29.2%, (2) 38.3%, (3) 50% and (4) 60% saturation with ammonium sulphate. The precipitates were dissolved in 0.005M-sodium pyrophosphate-HCl, pH 8.0 and dialysed against this buffer overnight at 2°. Fraction (3) had the best purity, giving 9.8 units of specific activity 1.05. This fraction was centrifuged at 259000g for 45 minutes, and the supernatant used for the following studies. Once again, a hard yellow pellet was found at the bottom of the cell after centrifugation.

When the supernatant from fraction (3) was subjected to starch gel electrophoresis, it became apparent that in this case enzyme activity was not associated with the main protein band observed in (i) above. However, this protein component was still present. Instead, most of the transferase moved with a lower mobility, and could not be allocated to any clear protein band. As shown in figure I-6 a second less marked transferase component migrated with a lower mobility than the main enzyme band. Here the protein pattern is shown on the right

Fig. I-6

Starch gel electrophoresis of ATP:NMN adenylyltransferase preparations.

(see text)

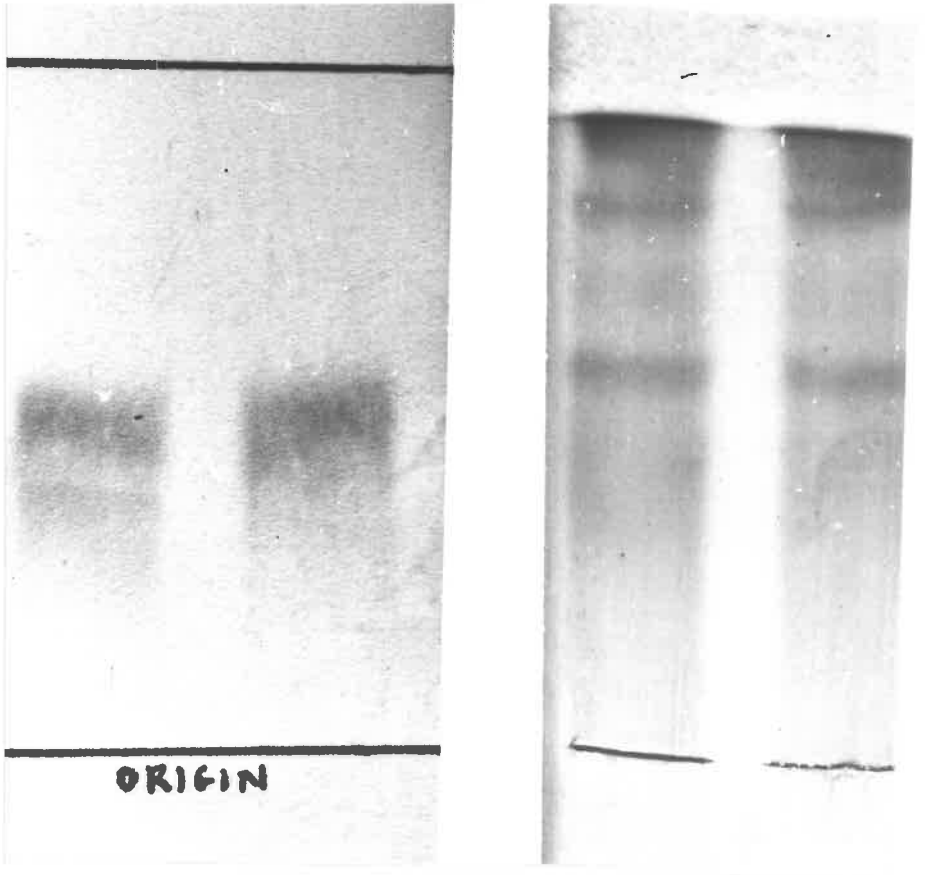


Fig. I-6

of the photograph. Both slots contain the same material.

(iii) To check that there is a real difference in mobility between the preparation used in (i) and that used in (ii), starch gel electrophoresis was carried out using three slots (figure I-7). In the left hand slot is fraction (3) (section (ii), above). The middle slot has a mixture of (ii)(fraction 3) and that used in (i) (a 1:1 mixture). That used in (i) occupies the right hand slot. All three solutions were dialysed against 0.005M-sodium pyrophosphate-HCl, pH 8.0 before application to the gel. As can be seen in figure I-7, there is a real difference in mobilities of the tranferase in (i) and (ii). The left hand portion of figure I-7, in which incisions can be seen in the gel at positions of transferase activity shows the protein pattern. The results suggest that fraction 3 (ii) is far from pure at a specific activity of 1.05. A total of three regions of pyrophosphorylase activity are easily discernible. The enzyme components with the greatest and least mobilities are associated with definite protein regions, but the band of transferase with an intermediate mobility migrates just ahead of one of the more minor protein bands.

(iv) Fraction 3 (ii) after centrifugation at 259000g, was subjected to starch gel electrophoresis in which a continuous buffer system of borate, pH 8.25, was substituted for the discontinuous buffer system used above. This resulted in a power resolution, both protein and transferase activity appearing to 'streak' out from the origin towards the anode. It would seem that the phenomenon seen in figure I-4 (a) is a result of the use of a continuous buffer

Fig. I-7

Starch gel electrophoresis of ATP:NMN adenylyltransferase preparations

(see text)

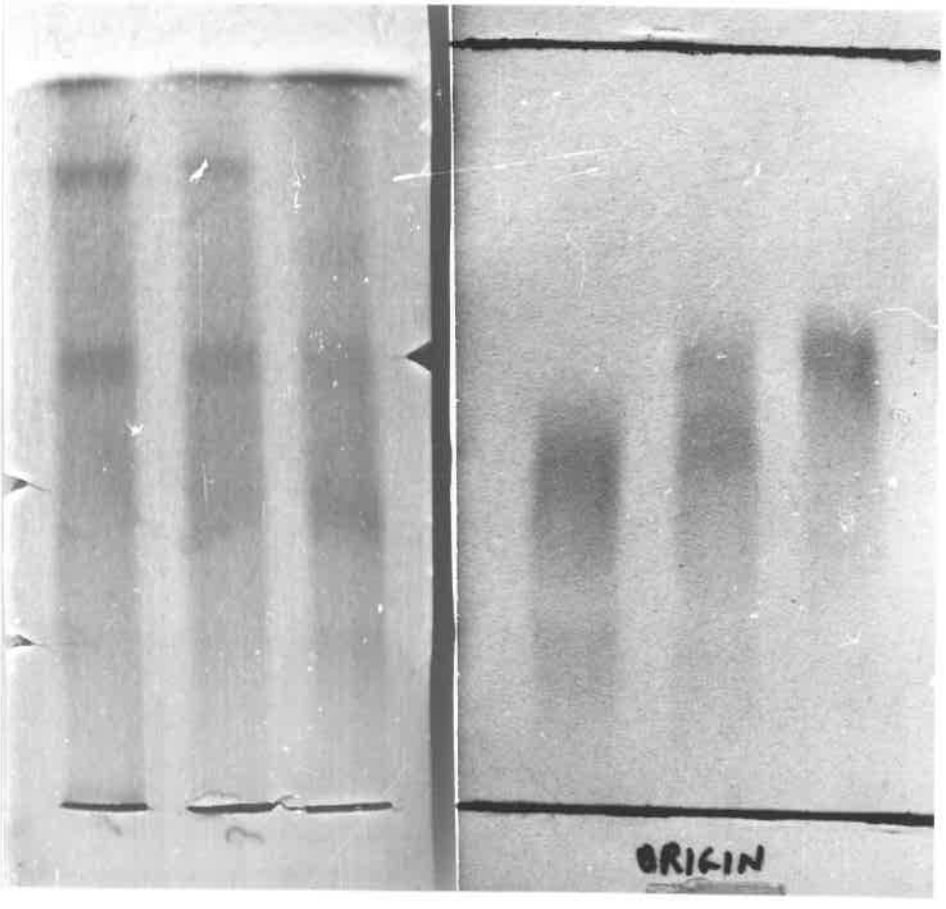


Fig. I-7

system, giving a diffuse distribution of components. High molecular weight material, present in the preparations not centrifuged at 259000g is probably not able to move freely in the gel.

D. Discussion

The formation of gels from nuclei has been studied in some detail by Dounce and his coworkers. It was found that gels are formed in the presence of dilute alkali, pH 9 to 10, or in molar saline solutions, pH 7 to 8, (Dounce, Witter and Monty, 1955); (Dounce and Monty, 1955). This gel arising from the contents of the nucleus is related to the formation of a 'halo' around the nucleus in the presence of molar sodium chloride solution, presumably due to the swelling of DNA through the nuclear periphery (Philpot and Stanier, 1956). Another agent known to produce gels from nuclear preparations is the detergent, sodium dodecyl sulphate. At a concentration of 0.41%, this latter compound, in bringing about gel formation, extracts very nearly 100% of the DNA from the nucleus (Kay, Simmons and Dounce 1952). It is believed that for this gel formation to take place, the deoxyribonucleoprotein structures must be intact and that mitochondrial deoxyribonuclease inhibits gel formation (Dounce, O'Connell and Monty 1957).

Some of the most effective agents in extracting ATP:NMN adenylyltransferase from liver nuclei do so with the formation of gels as above. Thus molar sodium chloride has been found to extract most of the transferase from liver nuclei (Hogeboom and Schneider, 1952),

and the present work shows that detergent is capable of doing the same. The use of 0.2M-disodium phosphate solutions in the routine extraction of this enzyme from the pig liver 'nuclear' fraction, described here as an initial step in its purification, also leads to the formation of a gel. There would thus seem to be little doubt that the transferase is intimately associated with the contents of the cell nucleus, as suggested by the work of Hogeboom and Schneider (1952), and Branster and Korton (1956). Further confirmation, and possibly an identification of transferase activity with some of the structures of the nucleus, may result if the method described here for the demonstration of transferase on starch gel blocks can be applied to its histochemical detection in tissues.

Prior to the extraction of the transferase from the 'nuclear' fraction with 0.2M-disodium phosphate solution for further purification, the standard practice of washing the nuclei in 0.14M-sodium chloride solutions has been adopted here. It was thought that this treatment would raise the specific activity of the final extract by removing some unwanted proteins from the still intact nuclei. Saline solutions of this concentration have been shown to remove approximately 42% of the nuclear protein from nuclei isolated in organic solvents from calf thymus, leaving the nuclei intact (Kirkham and Thomas, 1953). In addition, Roodyn (1956) has demonstrated that 90 to 95% of the total nuclear aldolase can be extracted from nuclei, isolated in aqueous media, with 0.14M-sodium chloride. In the present studies, a large proportion of the transferase was found to have escaped from the nuclei when washed with solutions containing salt of about this

concentration (Table I-6). It is thought that most of this is due to mechanical disruption of some of the nuclei in stirring the suspension (c.f. Hogeboom and Schneider, 1952). At the end of this treatment, most of the nuclei appear to be intact, and have clumped together, as noted by Kirkham and Thomas (1953).

The present work has shown that many techniques can be applied in purifiying the enzyme from 'nuclear' extracts. It is suggested that the most reliable method is to work up the phosphate extract of the 'nuclear' fraction according to Table I-7, giving a preparation with a specific activity of approximately 0.5 μmoles NAD synthesized per minute per mg. protein. This can be chromatographed on DEAE cellulose at pH 6.5, thus removing most of the nucleic acid and improving the specific activity somewhat (Table I-9). An acetone fractionation will then yield some transferase with a specific activity of approximately 3.0 (Table I-10 and I-11). If it is assumed that pig liver contains 20% protein on a wet weight basis (Spector, 1956), then the specific activity of ATP:NMN adenylyltransferase in fresh pig liver is approximately 0.0017 μmoles NAD synthesized per minute per mg. protein (Table I-6). This is similar to the specific activity of an extract of acetone powder in phosphate buffer (Table I-1). A specific activity of 3 would thus represent a purification of about 2,000 fold from pig liver. Previously a method has been given for obtaining transferase of specific activity 0.3 from pig liver nuclear extracts (Atkinson, Jackson and Morton, 1961). Kornberg (1950), has purified the enzyme to a specific activity of 0.21 from an acetone powder of pig liver. The yeast enzyme has been purified to a

specific activity of 7.55 (Kornberg, 1950).

The histochemical technique described above for the detection of ATP:NMN adenylyltransferase on starch gel is essentially an extension of the method outlined by Markert and Moller (1959) for dehydrogenases, except that paper and not agar was used to carry the necessary reagents. In addition, the use of nitroblue tetrazolium instead of neotetrazolium dispenses with the need for a catalytic amount of a redox dye (e.g. methylene blue), which served as an intermediate in the transfer of electrons from diaphorase to the tetrazolium salt (Machlas, Walker and Seligman, 1958). This is because nitroblue tetrazolium, due to its nitro group, has a much greater ability to serve as an electron acceptor compared to other tetrazolium salts (Tsou, et al. 1956).

In combination with the detection of protein on starch gel with amido black solutions, this histochemical method suggested that at a specific activity of about 1.0, the ATP:NMN adenylyltransferase purified from pig liver nuclei, is far from pure. The same deduction was made from experiments in which transferase activity was determined by direct assay of segments of the starch gel block (figure I-4). However, some caution is needed in accepting these conclusions, as it has been shown that crystalline yeast alcohol dehydrogenase, which gave only one major protein peak in the analytical ultracentrifuge, gives 18 protein components on starch gel electrophoresis using a discontinuous buffer system; only 5 of these had dehydrogenase activity (Watts, Donninger and Whitehead, 1961; Watts and Donninger, 1962).

On starch gel electrophoresis of ATP:NMN adenylyltransferase preparations using a discontinuous buffer system, a total of three regions of enzyme activity were observed (figure I-7). Two of these corresponded with protein components and the other could not be allocated to any distinct protein band. In addition the mobility of transferase components in two different preparations, one prepared six months beforehand and the other just before the electrophoresis was carried out, was quite different. A one to one mixture of the two ran just as expected from the electrophoretic behaviour of each alone. Although this may suggest the existence of multiple molecular forms (iso-enzymes) of the transferase, one cannot rule out altogether the possibility of denaturation or some related phenomenon giving rise to these iso-enzymes during the purification procedure, or some rather strong interaction of the transferase with other proteins in the preparation leading to formation of complexes which are not broken up during starch gel electrophoresis. Many enzymes have been shown to exist in multiple forms as indicated in a monograph edited by Wroblewski (1961), and it seems that the transferase from pig liver nuclei is no exception in this regard. This may explain the occurrence of multiple peaks of transferase activity in the eluate from DEAE cellulose, pH 7.5 (figure I-2), and the difficulty encountered in further purifying the preparations obtained so far.

It was found that a discontinuous buffer system similar to that of Poulik (1957) gave a much better resolution of the transferase on starch gel electrophoresis, than did a continuous buffer system.

This aspect of starch gel electrophoresis has been investigated to some extent by Barret, Friesen and Astwood (1962), who find that it is the 'borate front' (as yet not a fully explained phenomenon) which appears to be a major factor in obtaining a high degree of resolution and separation. The individual components are sharpened by the 'borate front' as it passes, and the mobility of all anionic bands increased at this time.

The stability of transferase preparations towards urea may eventually prove useful in further purification. Other enzymes have been found to be stable towards urea solutions, including ribonuclease (Anfinson et al., 1955), pepsin (Steinhardt, 1938; Perlman, 1956) and trypsin (Harris, 1956). Pyruvic kinase (Morawiecki, 1960) and chymotrypsin (Harris, 1956) are among those enzymes which lose enzymic activity in urea solutions. It is not known as yet if ATP:NMN adenylyl-transferase, like ribonuclease and unlike trypsin, is fully active in urea solutions, but it certainly retains its activity after removal of urea by dialysis.

II

Specificity of ATP:NMN Adenylyltransferase with Respect to NMN Analcgues.

This section describes the preparation and properties of a number of analogues of nicotinamide nucleotide. The analogues were compared with NMN as adenylyl acceptors in the presence of ATP and the transferase, they also were tested as inhibitors of adenylyl transfer to NMN.

A. Materials

Nucleotides ATP and NAD were purchased from Sigma Chemical Co., and thionicotinamide adenine dinucleotide (lot number 4601), 3-acetylpyridine adenine dinucleotide (lot number 2702) and pyridine-aldehyde adenine dinucleotide (lot number 3301) from Pabst Laboratories, Milwaukee, Wisconsin. NMN was prepared as previously described (Atkinson, Jackson and Morton, 1961). Synthetic samples of thionicotinamide mononucleotide, pyridine mononucleotide, 6-phosphoglucosyl nicotinamide and nicotinamide riboside were provided by Mr. R. Naylor. The method of Haynes et al. (1957) was used to prepare these analogues. Synthetic α-NMN was also provided by Mr. Naylor; this was prepared from tribenzoylribosylamine.

Buffers. Glycylglycine was obtained from B.D.H. (laboratory reagent grade) and tris (hydroxymethyl) amino methane from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Inorganic Reagents were all of analytical reagent grade.

NAD Pyrophosphatase was partially purified from potato by the method of Kornberg and Procer (1950). Essentially an ammonium sulphate fractionation was used, followed by two successive fractionations with ethanol. The final product was dissolved in water and stored at -15°.

ATP:NMN adenylyltransferase was prepared from pig liver nuclear extracts according to the method outlined in Table I-7. Before use, the preparation, of specific activity

0.54 μmoles of NAD synthesized per minute per mg. of protein, was dialysed against 0.01M-sodium phosphate, pH 7.5, and diluted with water.

B. Methods

Determination of the rate of adenylyltransfer from ATP to NMN and its analogues.

Three methods were used to determine rates in this system: (a) a non-coupled assay, where the substrates were incubated with transferase, the reaction was stopped and the dinucleotide formed was estimated with yeast alcohol dehydrogenase; (b) a coupled assay, in which ethanol and yeast alcohol dehydrogenase was included in the incubation mixture together with the transferase and its substrates, enabling a direct estimation of dinucleotide formation; (c) estimation of pyrophosphate formed after incubation of the transferase with its substrates as in (a). Procedure (c) was adopted where nicotinic acid mononucleotide was used as substrate. This method is necessary in this case, as nicotinic acid adenine dinucleotide is not reduced by yeast alcohol dehydrogenase (Lamborg et al., 1958; van Eys et al., 1958)

(a) Non-coupled assay. NMN or its analogues were incubated with the transferase in the presence of 100 mM-glycylglycine, pH 8.0, 4 mM-ATP and 10 mM-$MgCl_2$ at 37°. The total volume of the mixture was 1.0 ml. After an appropriate time interval, the reaction was stopped by the addition of 1.5 ml. of 0.5M-trichloroacetic acid, and the small amount of precipitated protein was centrifuged to the bottom of the assay tube. 2.0 Millilitres if the supernatant was

added to a spectrophotometer cuvette of 4 cm. light path containing 3.0 ml. of a solution of 0.835M-ethanol - 0.417M-glycine - 0.416M-sodium hydroxide. The amount of NAD, or its analogues, present in the mixture, now at a pH of 9.5, was estimated by the difference in optical density of the solution at the wavelength of maximum absorption of the reduced dinucleotide before and after the addition of 200 γ of yeast alcohol dehydrogenase contained in 0.02 ml. For reduced NAD, a millimolar extinction coefficient of 6.22 at 340 mμ was used (Horecker and Kornberg, 1948); for reduced thionicotinamide adenine dinucleotide the millimolar extinction coefficient used was 11.3 at 395 mμ, for reduced 3-acetylpyridine adenine dinucleotide 9.1 at 363 mμ, and for reduced 3-aldehydepyridine adenine dinucleotide 9.3 at 358 mμ (Pabst Laboratories, 1961).

(b) Coupled assay. The coupled enzymic reaction was initiated by the addition of 0.01 ml. (or 0.02 ml. where NMN analogues were present) of an ATP:NMN adenylyltransferase preparation containing 0.027 units (or 0.054 units) of enzyme to 2.795 ml. of a solution containing 3.6mM-$MgCl_2$, 0.72mM-ATP, 0.3mM-NMN or analogue, 75 γ of yeast alcohol dehydrogenase, 620mM-ethanol and 143mM-Tris, pH 9.5. The reaction was carried out in a spectrophotometer cuvette of 1 cm. light path. The formation of reduced NAD or analogue was observed from the increase of optical density of the solution in the region 300 to 450 mμ. A recording DK2 Beckman spectrophotometer was used for this purpose; measurements were carried out at 25°.

(c) Assay by pyrophosphate determination.

Nicotinic acid mononucleotide or NMN was incubated with transferase, 100mM-glycylglycine, pH 7.6, 5mM-ATP and 15 mM-$MgCl_2$ at 37° for four minutes. The total volume of the reaction mixture was 1.0 ml. The enzymic reaction was stopped by adding 1.5 ml. of 0.5M-trichloroacetic acid, and the precipitated protein was centrifuged to the bottom of the tube. 2.0 Millilitres of the supernatant was added to a small tube containing 100 mg. of acid-washed charcoal (Norite SX-30), and the mixture was shaken to aid the adsorption of the nucleotides onto the charcoal. The contents of the tube was then poured into a small sintered funnel, and the filtrate was collected in a 10 ml. standard flask. The tube was rinsed with 2x2 ml. of water, and this was filtered through the same sinter into the flask. When a series of such filtrates from various incubation mixtures had been collected in this manner, 2.0 ml. of a molybdate-hydrazine solution was added to each flask, and the volume made up to 10.0 ml. The molybdate-hydrazine solution was prepared by mixing a 1% ammonium molybdate solution (in 5N-sulphuric acid) with 0.25% aqueous hydrazine sulphate solution in the ratio of 4 to 1 just before the experiment was to be carried out. The flasks were then heated at 100° for 30 minutes, cooled, mixed, and the optical density of the solutions they contained measured at 825 mμ. Internal pyrophosphate standards were employed by adding suitable volumes of a 1mM-pyrophosphate solution to similar incubation mixtures. This method is based on Boltz and Mellon's (1947) method for the analysis of orthophosphate.

C. Results

(a) Preparation of thionicotinamide mononucleotide, 3-acetylpyridine mononucleotide and 3-pyridinealdehyde mononucleotide.

100 Milligrams of thionicotinamide adenine dinucleotide was dissolved in 10 ml. of water, and the solution was adjusted to pH 7.0 with 4N-potassium hydroxide solution. After the addition of 1 ml. of a NAD-pyrophosphatase preparation, the pH of the solution was maintained near neutrality by the automatic addition of 4N-potassium hydroxide using a type TTT 1 titrator in combination with a type SBR 2 titrograph (Radiometer, Copenhagen). The reactants were kept at 25°. The enzyme preparation added was capable of hydrolysing 66 μmoles of NAD per ml. per hour at 37°, when assayed according to the method of Kornberg and Pricer (1950). The hydrolysis of the dinucleotide was complete after 7.67 hours at 25°, by which time 0.12 ml. of 4N-potassium hydroxide had been added. Cold 72% perchloric acid solution (2 ml.) was added to the reaction mixture and the resultant suspension was centrifuged at 10000g for 30 minutes. The pH of the supernatant was adjusted to 7.0 with 4N-potassium hydroxide solution, and the mixture allowed to stand at 2° for 24 hours to allow complete precipitation of insoluble potassium perchlorate. The precipitate was filtered off, leaving 22.5 ml. of filtrate. Ascending chromatography in butanol-acetic acid - water (20 : 3 : 7 ; vols) and paper electrophoresis in 2% formic acid showed that the filtrate, in addition to thionicotinamide mononucleotide, contained traces of thionicotinamide riboside, and

substantial amounts of adenylic acid, adenosine and adenine. The mononucleotide was purified by chromatography on Amberlite CG-400 (chloride form, dimensions of the column: 2.3 cm. diameter x 5.4 cm. long) and then on Dowex-50 (hydrogen form, dimensions: 1.4 cm. diameter x 3 cm. long). All contaminants which absorbed light at 254 mμ were removed by this procedure, the thionicotinamide mononucleotide moving freely through both resins. The final eluate was freeze dried, yielding a yellow solid, which was dissolved in water and stored at -15°. Assuming a millimolar extinction coefficient of 6.0 at 266 mμ (obtained by Mr. R. Naylor on synthetic material; see below), spectrophotometric analysis of the solution showed a yield of 31% of the mononucleotide from thionicotinamide adenine dinucleotide.

3-Acetylpyridine mononucleotide and 3-pyridinealdehyde mononucleotide were prepared in the same manner, starting from 100 mg. of dinucleotide. It was found that 2 ml. of the NAD-pyrophosphatase preparation was sufficient to completely hydrolyse these dinucleotides in 3.75 and 4.5 hours respectively, 0.1 ml. of 4N-potassium hydroxide solution being consumed in the process. Both the above nucleotides were obtained in a 45% yield from their respective dinucleotides, on the basis of a millimolar extinction coefficient of 4.6 (see below) at the absorption maximum in 0.1M-sodium phosphate solutions, pH 7.5.

The time taken for the complete hydrolysis of thionicotinamide adenine dinucleotide, 3-acetylpyridine adenine dinucleotide and 3-aldehydepyridine adenine dinucleotide was approximately the same as that for equivalent amounts of NAD, indicating the broad specificity of the nucleotide pyrophosphatase preparation from potato. With all

these substrates, including NAD, considerable amounts of adenosine and adenine were obtained during hydrolysis by this particular preparation. Four previous preparations from potato, obtained in the same way, did not show the formation of these byproducts when incubated with NAD.

(b) Preparation of nicotinic acid mononucleotide

The nucleotide was made by a modification of Atkinson and Morton's (1960) method. 0.95 Gram of NAD was freeze-dried in a 20 ml. tube and the residue was covered with 10 ml. of dry acetic acid. The contents of the tube were frozen by immersion in a cellosolve-dry ice mixture and approximately 10 ml. of dry nitrous anhydride, generated from the reaction of 50 ml. of concentrated nitric acid on 50 g. of arsenious oxide, was condensed into the tube. The tube was kept at room temperature for one hour, in which time the nitrous anhydride had penetrated through the thawing acetic acid and reached the NAD at the bottom of the tube. This resulted in the evolution of gas; this ceased after a further 30 minutes. The acetic acid and nitrous anhydride were removed by passing a stream of dry air through the tube, and finally by freeze-drying. The residue was dissolved in water and titrated to pH 7.0 with 4N-potassium hydroxide solution.

To this solution 10 ml. of a nucleotide pyrophosphatase preparation was added, and the solution was kept at pH 7 by the automatic addition of 4N-potassium hydroxide as before. After 50 hours at 25°, hydrolysis appeared to be complete. 3.0 Grams of solid trichloroacetic acid was dissolved in the mixture, and the precipitated protein removed by centrifugation at 10,000g for 30 minutes. The supernatant (50 ml.) was extracted with 3 x 50 ml. of ether, and the aqueous phase

brought to pH 8.0 with a solution of dilute ammonia. The resulting solution was applied to a column of Dowex-2 formate (3.1 cm. diameter x 13.5 cm. long), and the resin was eluted successively with water, 0.1N-, 0.25N-, 1.0N-, and 2.0N- formic acid solutions. Nicotinic acid riboside (250 μmoles ; for details see below), appeared on elution with water, followed by some hypoxanthine derivatives, which were not characterized. Nicotinic acid mononucleotide (262 μmoles) was eluted from the resin with 1.0N-formic acid, and was preceded by a mixture of this nucleotide (330 μmoles) and a hypoxanthine derivative. An overall recovery of 70% of pyridine derivatives was achieved. Before use in the enzymic experiments described below, the mononucleotide was further purified by passage through a column of Dowex-50 resin (H^+ form).

Atkinson and Morton (1960) did not observe the formation of nicotinic acid riboside in a similar experiment. This compound, which had the same spectrum as its 5'-phosphate in 0.1M-sodium phosphate, pH 7.5 or M-potassium cyanide had the same R_f (0.16) in butanol-acetic acid-water (20:3:7; vols) as a synthetic sample provided by Mr. R. Naylor. The riboside may have been formed by enzymic hydrolysis during the long incubation with the potato enzyme or by acid hydrolysis through inadequate drying of the starting materials. The procedure described here proved to be less convenient than Atkinson and Morton's (1960) synthesis of nicotinic acid nucleotide directly from NMN as this does not involve separation from hypoxanthine derivatives.

(c) Characterization of NMN analogues

Each nucleotide was found to move as a single spot on paper chromatography. The R_f values for the nucleotides in two solvent systems are shown in Table II-1. The nucleotides were shown to have

Table II-1

Chromatography of NMN analogues.

Nucleotide	R_f in butanol/acetic acid/water (20:3:7; vols.)	R_f in isobutyric acid/conc. NH_3/water (66:1:33; vols.) (2 days after mixing)
Nicotinic acid mononucleotide	0.019	0.30
3-pyridinealdehyde mononucleotide	0.099	0.32
nicotinamide mononucleotide	0.037	0.43
thionicotinamide mononucleotide	0.102	0.49
3-acetylpyridine mononucleotide	0.056	0.52

The chromatograms were developed over a period of 10 hours, using the ascending technique.

the same mobility as NMN on paper electrophoresis in 2% formic acid solution, with the exception of nicotinic acid mononucleotide which migrated towards the anode. NMN under these conditions remains

in the vicinity of the point of application. Thionicotinamide mononucleotide, like NMN, was shown to fluoresce after exposure to an atmosphere of methyl ethyl ketone - ammonia (Carpenter and Kodicek, 1950).

The ultraviolet absorption spectra of these nucleotides in 0.1M-sodium phosphate, pH 7.5 and in aqueous molar potassium cyanide is shown in figures II-1 to II-4. As expected, they all show an absorption maximum near 266 mμ in phosphate buffer, and a maximum at higher wavelengths in cyanide. Their spectral characteristics are recorded in Table II-2. Millimolar extinction coefficients of 6.0 for the thioamide nucleotide and 4.6 for the other nucleotides at their maxima were derived from values found by Mr. R. Naylor for analytically pure samples of corresponding ribosides. Nucleotides usually have the same spectra as the corresponding nucleosides (Siegal, Montgomery and Bock, 1959). NMN also has a millimolar extinction coefficient of 4.6 at 265.5 mμ (Atkinson et al., 1961). The concentrations and the extinction coefficients of the nucleotides in cyanide were calculated on this basis.

In aqueous molar cyanide the spectra of these nucleotides is relatively unstable. This has been noted previously for NMN (Jackson, 1960). In general the highest optical density reading at the absorption maximum is quickly attained, and then falls off slowly with time. The time at which the highest optical density measurement is obtained after adding the nucleotide to cyanide is noted in Table II-2. The extinction coefficient for the absorption maximum was calculated from the optical density at this time. There is also a

Fig. II-1

U.V. spectrum of thionicotinamide mononucleotide

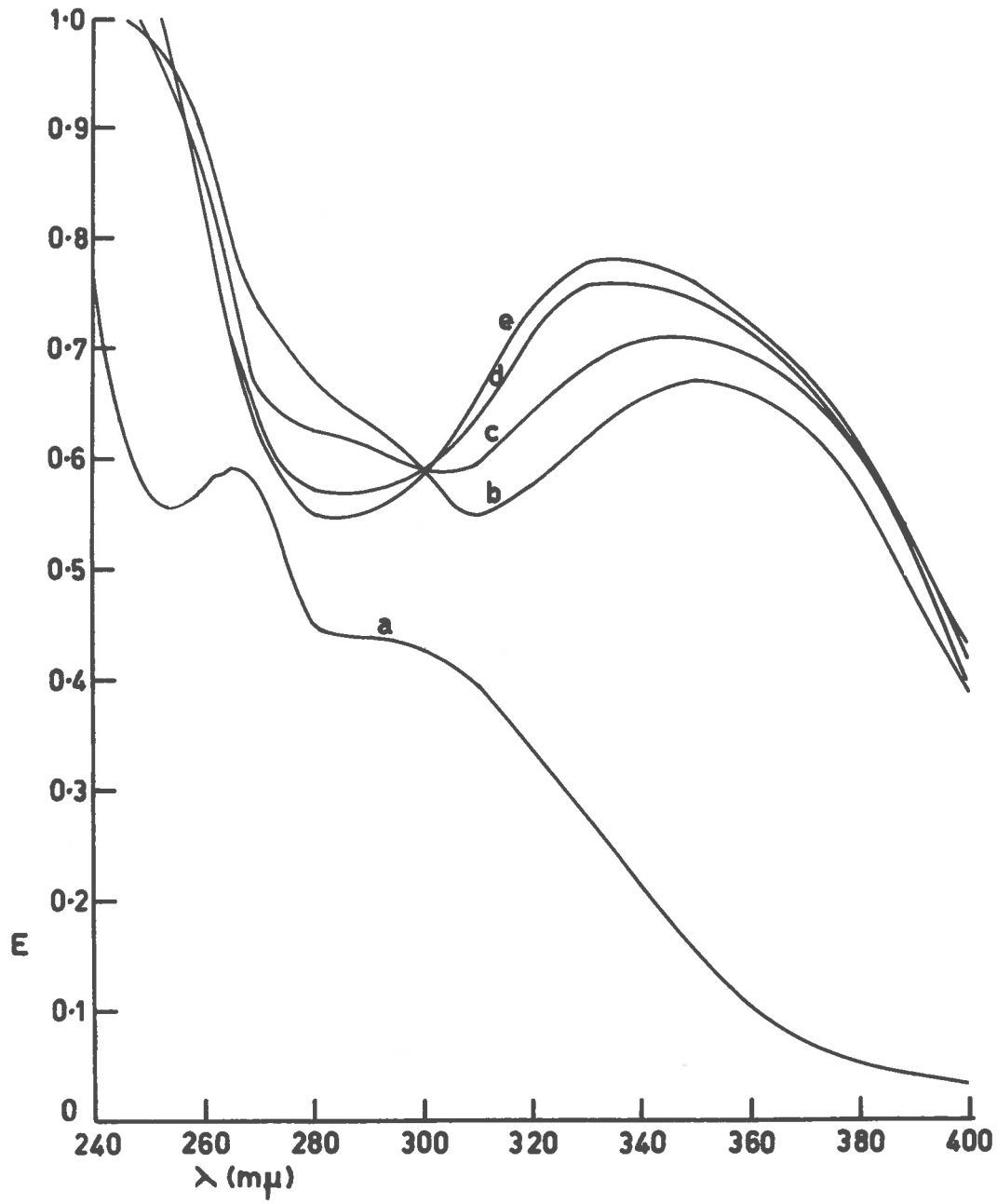
(a) in 0.1M-sodium phosphate, pH 7.5

(b) in M-KCN after 60 minutes

(c) after 30 minutes

(d) after 11 minutes

(e) after 6 minutes 40 seconds



U.V. Spectrum of Thionicotinamide Mononucleotide

Fig. II - 1

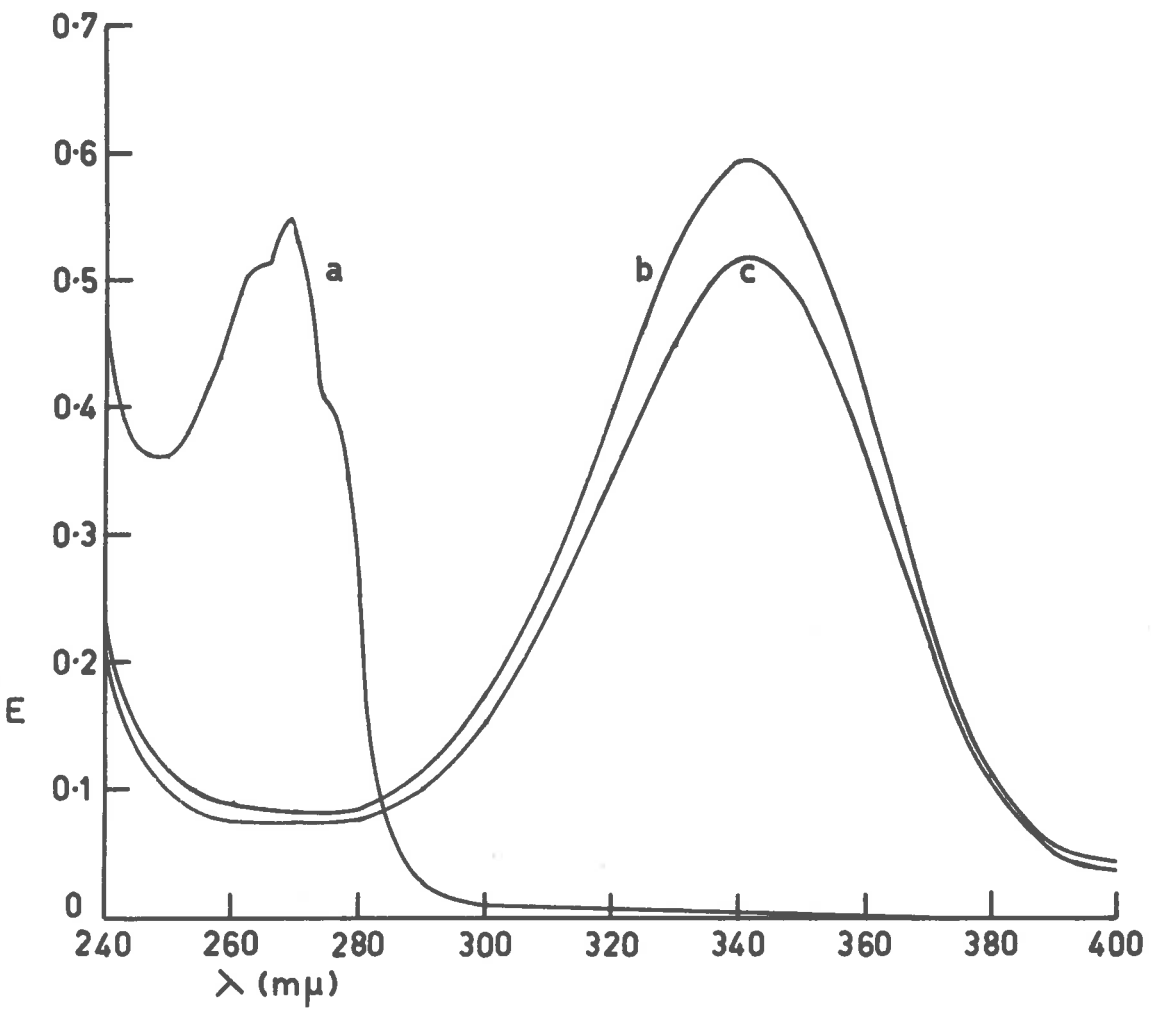
Fig II-2

U.V. spectrum of acetylpyridine mononucleotide

(a) in 0.1M-sodium phosphate, pH 7.5

(b) in M-KCN after 2.5 minutes

(c) after 30 minutes



U.V. Spectrum of Acetylpyridine Mononucleotide

Fig. II - 2

Fig II-3

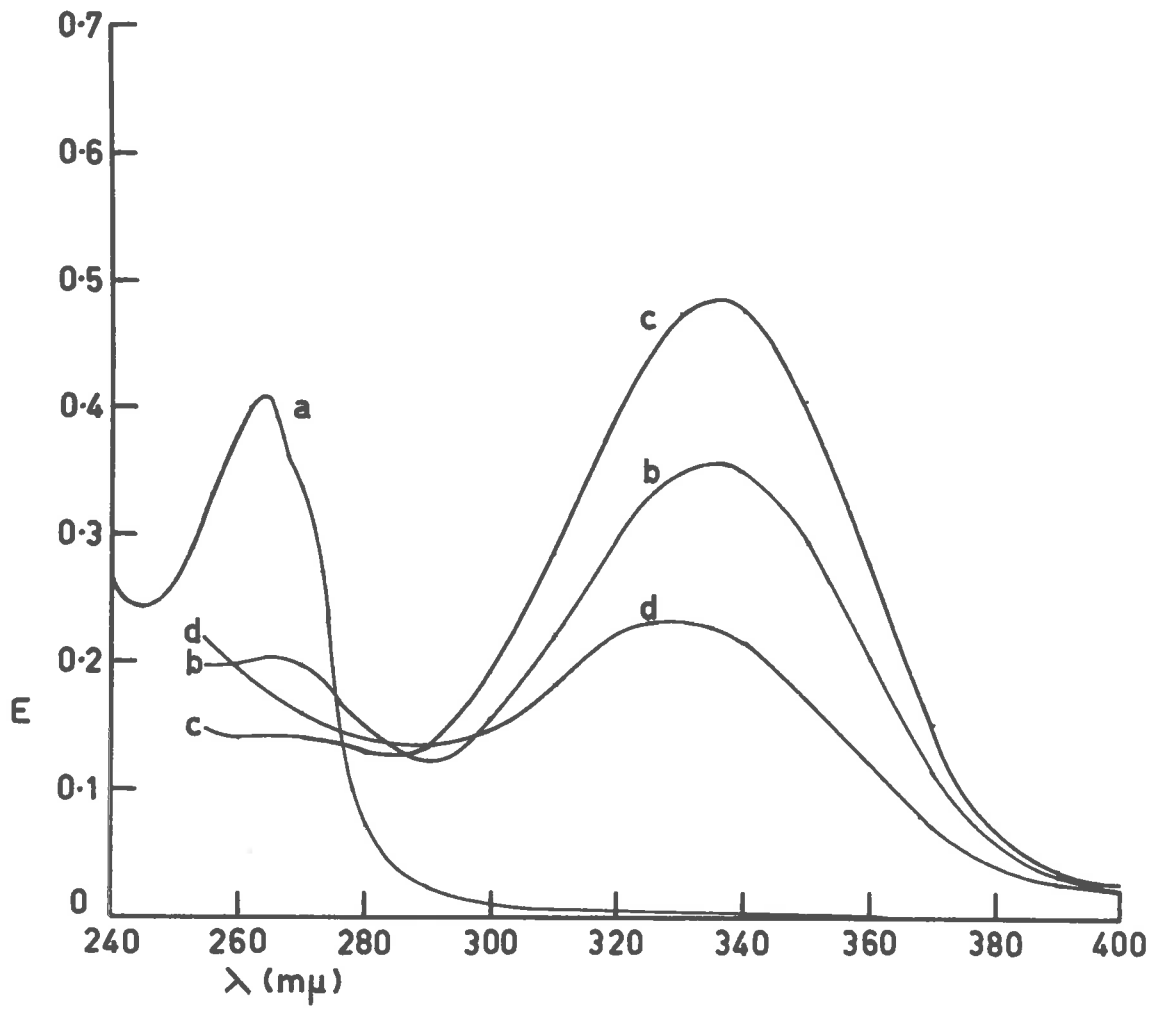
U.V. spectrum of pyridine aldehyde mononucleotide

(a) in 0.1M-sodium phosphate, pH 7.5

(b) in M-KCN after 2 minutes 33 seconds

(c) after 6 minutes

(d) after 46.5 minutes



U.V. Spectrum of Pyridinealdehyde Mononucleotide

Fig. II - 3

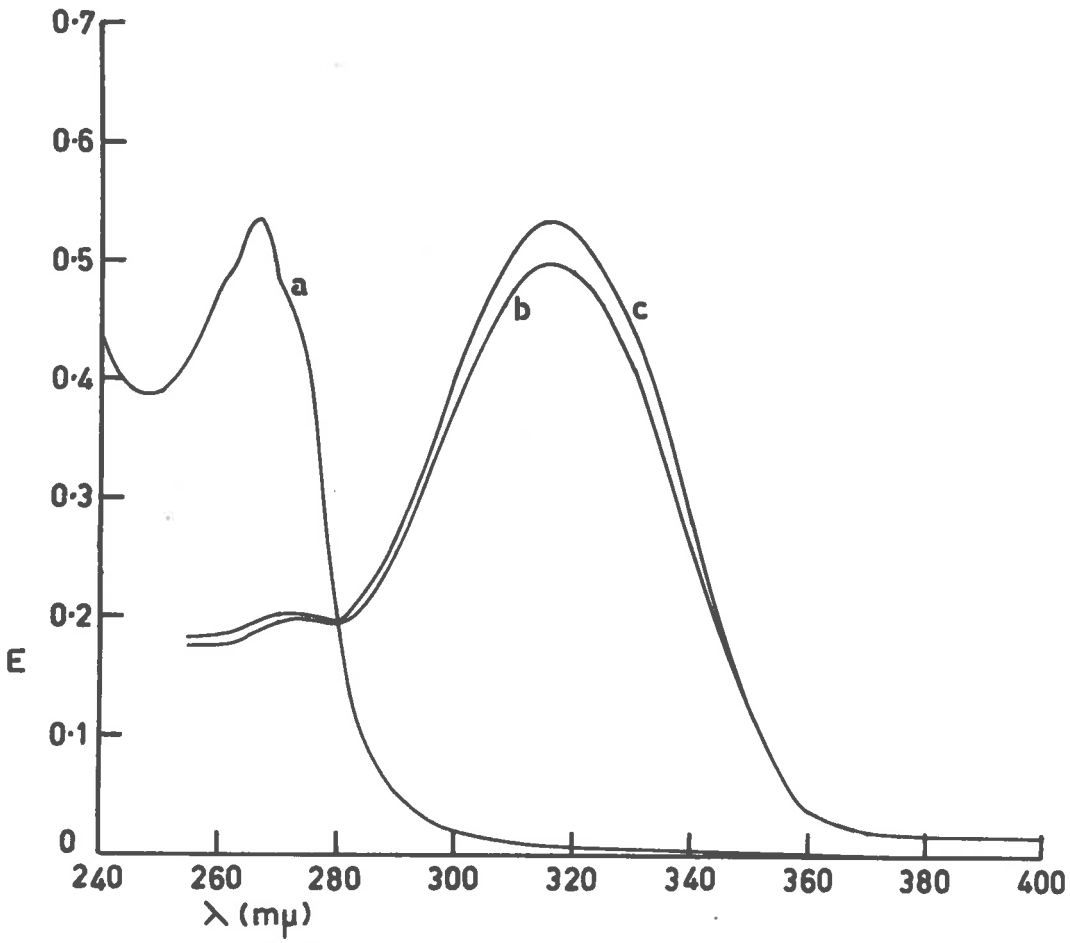
Fig. II-4

U.V. spectrum of nicotinic acid mononucleotide

(a) in 0.1M-sodium phosphate, pH 7.5

(b) in M-KCN after 3 minutes

(c) after 6 to 25 minutes.



U.V. Spectrum of Nicotinic Acid Mononucleotide

Fig. II - 4

shift at the wavelength of the absorption maximum concomitant with the fall in optical density. These phenomena probably account for the differences in the extinction coefficients in aqueous cyanide recorded in Table II-2 and those for the corresponding adenine dinucleotides noted in 'Ultraviolet Absorption Spectra of Pyridine Coenzymes and Coenzyme Analogues'(circular OR-18, Pabst Laboratories (1961)), where allowances may not have been made for the lack of stability of the spectrum in cyanide. Thus NAD in cyanide is recorded as having a millimolar extinction coefficient of 5.9 at 327 mμ, 3-acetylpyridine adenine dinucleotide 8.7 at 343 mμ, 3-aldehyde-pyridine adenine dinucleotide 4.6 at 317 mμ and thionicotinamide adenine dinucleotide 10.0 at 355 mμ.

It would seem that measurements of optical density in cyanide solutions is of little use in determining concentrations of these pyridine mononucleotides accurately. The ultraviolet spectrum of nicotinic acid mononucleotide is the most stable in cyanide, followed by nicotinamide-, 3-acetylpyridine-, thionicotinamide- and 3-aldehyde-pyridine mononucleotides in that order of decreasing stability.

(d) Preliminary experiments on substrate specificity

3-Acetylpyridine mononucleotide was shown to be a substrate for ATP:NMN adenylyltransferase by recording at various time intervals the spectrum of a solution containing the nucleotide (0.4mM) and the components of the coupled assay system (Methods (b)). The formation of reduced 3-acetylpyridine adenine dinucleotide was very slow. After 18 hours at 25° the greatest increase of absorption was at 363 mμ and this corresponded to the synthesis of 0.098 μmoles of reduced

Table II-2

U.V. Absorption properties of some NMN analogues

Nucleotide	0.1M-phosphate, pH 7.5			1M-KCN		
	shoulder (mμ)	λ max (mμ)	shoulder (mμ)	isosbestic point (mμ)	λ max (mμ)	Time after mixing (minutes)
Nicotinamide mononucleotide	261	265.5	272.5	307	325	3.5 - 7.0
mε		4.6			6.3	
Nicotinic acid mononucleotide	261	266.5	272.5	305	316	6.0 - 45.0
mε		4.6			4.6	
3-Acetylpyridine mononucleotide	264	268.5	276	280	341	2.5
mε		4.6			10.0	
3-Pyridinealdehyde mononucleotide	260	264	270	287	335	6.0 - 10.0
mε		4.6			5.4	
Thionicotinamide mononucleotide	-	266	290	300	335	6.7
mε		6.0			7.9	

The wavelength data were taken from figures II-1 to II-4; mε represents the millimolar extinction coefficient (see text). The term isosbestic point represents the (only) wavelength at which no change in optical density occurs during decomposition of the cyanide adduct in aqueous cyanide. The fact that there is only one isosbestic point suggests that the changes observed in optical density at other wavelengths are due to a simple transformation into a second absorbing species of molecule.

dinucleotide. Under the same conditions 0.4mM-NMN gave rise to 0.15 μmoles of reduced dinucleotide in 18.5 minutes, indicating that the analogue reacts approximately 1% as fast as NMN at these concentrations. The same system only gave rise to a trace of reduced thionicotinamide adenine dinucleotide from 0.4mM thionicotinamide mononucleotide, and synthesis of reduced dinucleotide from 3-pyridinealdehyde mononucleotide was not apparent at this concentration.

It is possible that the lack of reactivity of the last of these nucleotides is due to a lower rate of reduction of the corresponding adenine dinucleotide by yeast alcohol dehydrogenase, compared with 3-acetylpyridine adenine dinucleotide. 3-Pyridinealdehyde- and 3-acetylpyridine adenine dinucleotides have been reported to be reduced by the dehydrogenase at 2% and 10% respectively of the rate of NAD reduction. The apparent K_m for the two dinucleotide analogues was shown to be the same as for NAD (Kaplan et al., 1956). At a concentration of 0.2mM, thionicotinamide adenine dinucleotide is reduced by the dehydrogenase at 16% of the rate of NAD reduction (Anderson and Kaplan, 1959).

To render the system independent of the rate of reduction of the NAD analogues by dehydrogenase, the mononucleotide analogues were incubated with ATP, $MgCl_2$ and transferase, the reaction stopped and the amount of dinucleotide analogue so formed estimated by reduction with dehydrogenase (Methods (a)). Table II-3 shows the results of such an experiment. The analogues were incubated for 45 minutes (37°) with 0.27 units of ATP:NMN adenylyltransferase (535 γ protein), and NMN for 5 minutes with 0.054 units of the same preparation.

The spectrum of the products of the reaction was recorded in the region of 300 to 400 mμ before and after the addition of 200 γ of yeast alcohol dehydrogenase. In each case there was maximal increase in absorption at a wavelength consistent with the spectrum for the reduced dinucleotide analogue concerned, indicating that all analogues tested can act as substrate for the nuclear transferase. 3-Pyridinealdehyde mononucleotide reacted with ATP at an extremely low rate, although the reaction was conclusively demonstrated. However, there is also a small increase in optical density over the region 300 to 400 mμ during reduction with dehydrogenase, which is not centred at the absorption maximum of 3-pyridinealdehyde adenine dinucleotide, but is spread over the entire region. This is possibly due to the formation of an alkali product of the mononucleotide, and confuses to some extent accurate measurements of dinucleotide formation in this case. At approximately equivalent concentrations, 3-pyridinealdehyde mononucleotide entered into the adenylyl transfer reaction at about one sixth the rate of 3-acetyl pyridine mononucleotide.

(e) Kinetic parameters for NMN and analogues in the transferase reaction

Using the non-coupled assay system (Methods (a)), apparent kinetic parameters for adenylyl transfer to 3-acetylpyridine mononucleotide and thionicotinamide mononucleotide where compared to those for NMN. For thionicotinamide mononucleotide, 535 γ of the transferase preparation was employed during a 30 minute incubation, for 3-acetylpyridine mononucleotide 267.5 γ for 30 minutes and for NMN

Table II-3

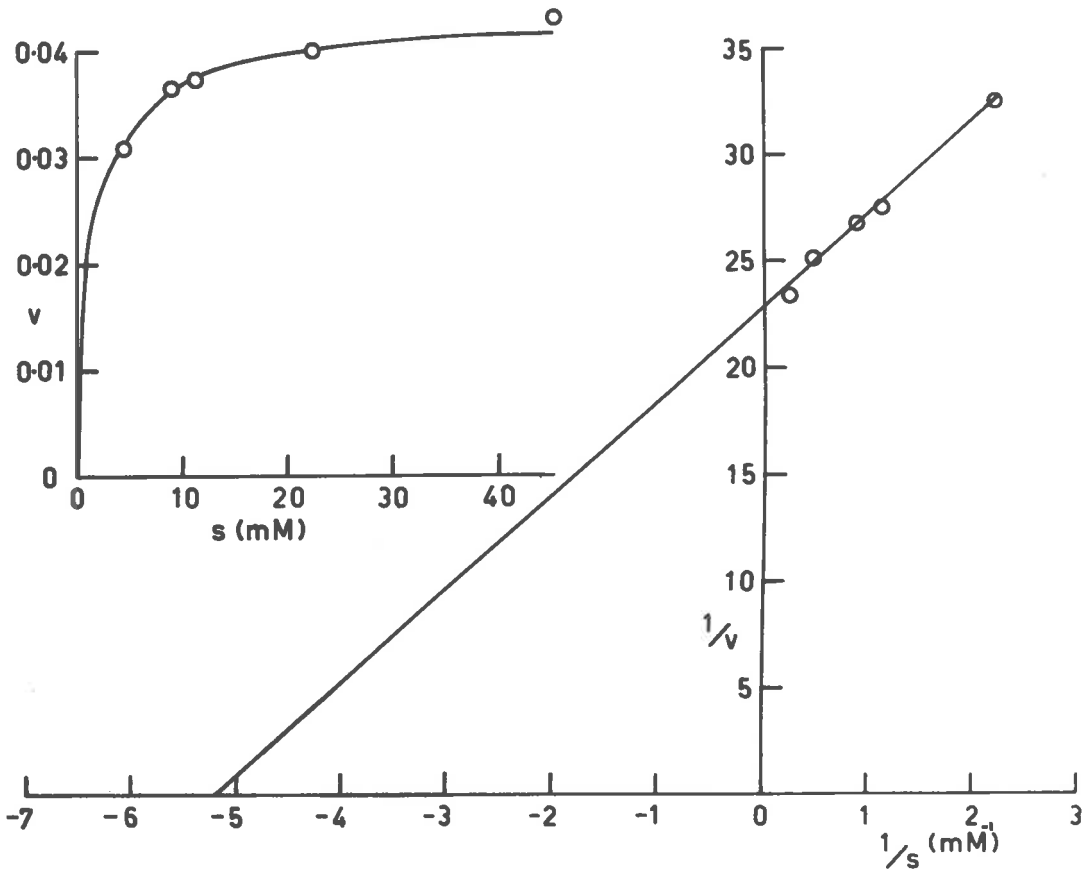
A Comparison of the ability of mononucleotide analogues to substitute for NMN in the ATP:NMN adenylyltransfer reaction.

Nucleotide	Concentration (mM)	λ max after reduction with dehydrogenase (mμ)	NAD or analogue formed per minute (μmoles)	Rate of dinucleotide formation compared with 2.22mM NMN (%)
Nicotinamide mononucleotide	0.44	340	0.158	76.3
	2.22	340	0.207	100.0
3-Acetylpyridine mononucleotide	2.82	363	0.0034	1.5
	5.65	363	0.0070	3.0
Thionicotinamide mononucleotide	0.575	395	0.002	1.0
3-Aldehydepyridine mononucleotide	2.12	c.a.360	< 0.0005	< 0.25

107 Y for 5 minutes. The results show that both analogues are very poor substrates compared to NMN (Table II-4 and figures II-5 to II-7). There is however, a striking difference between the two analogues in their kinetic behaviour. Although 3-acetylpyridine mononucleotide is capable of higher rates in the transfer reaction than is thionicotinamide mononucleotide, it has an extremely high K_m. This is in contrast to the thio analogue which has an apparent K_m only slightly more than four times that of NMN (Table II-4). A kinetic

Fig. II-5

Adenylyl transfer to NMN

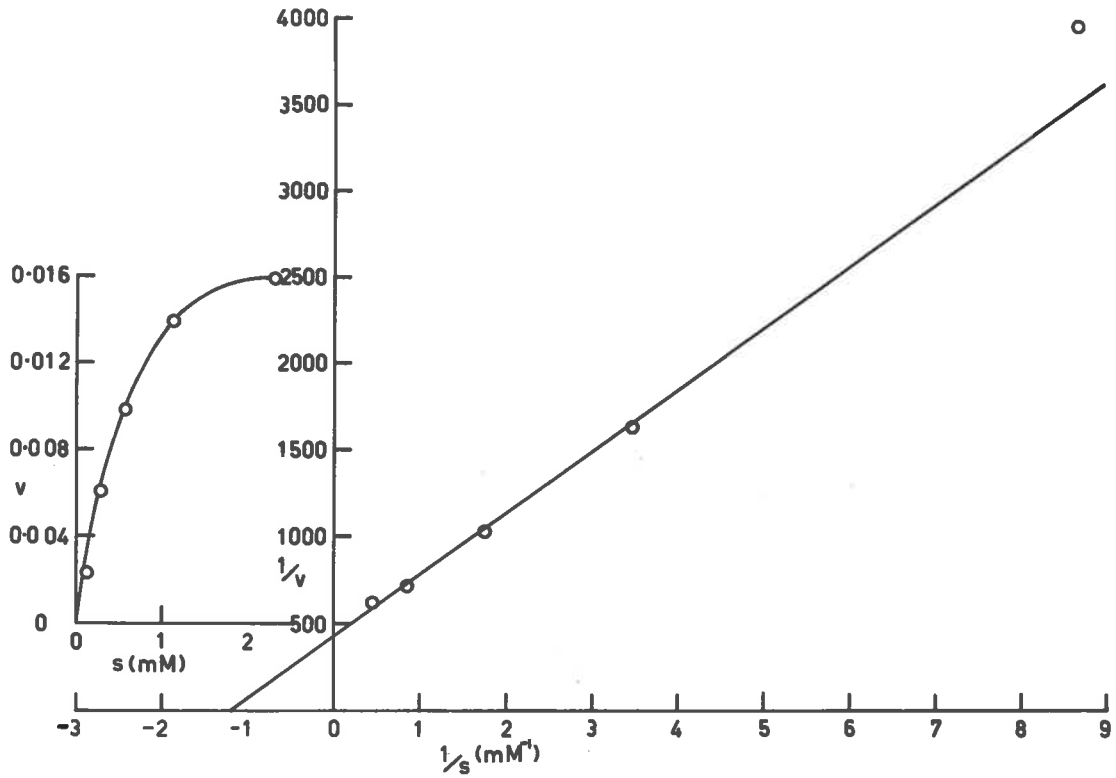


Effect of Mononucleotide Concentration
on the Transferase Reaction :- NMN

Fig. II - 5

Fig. II-6

Adenylyl transfer to thionicotinamide mononucleotide

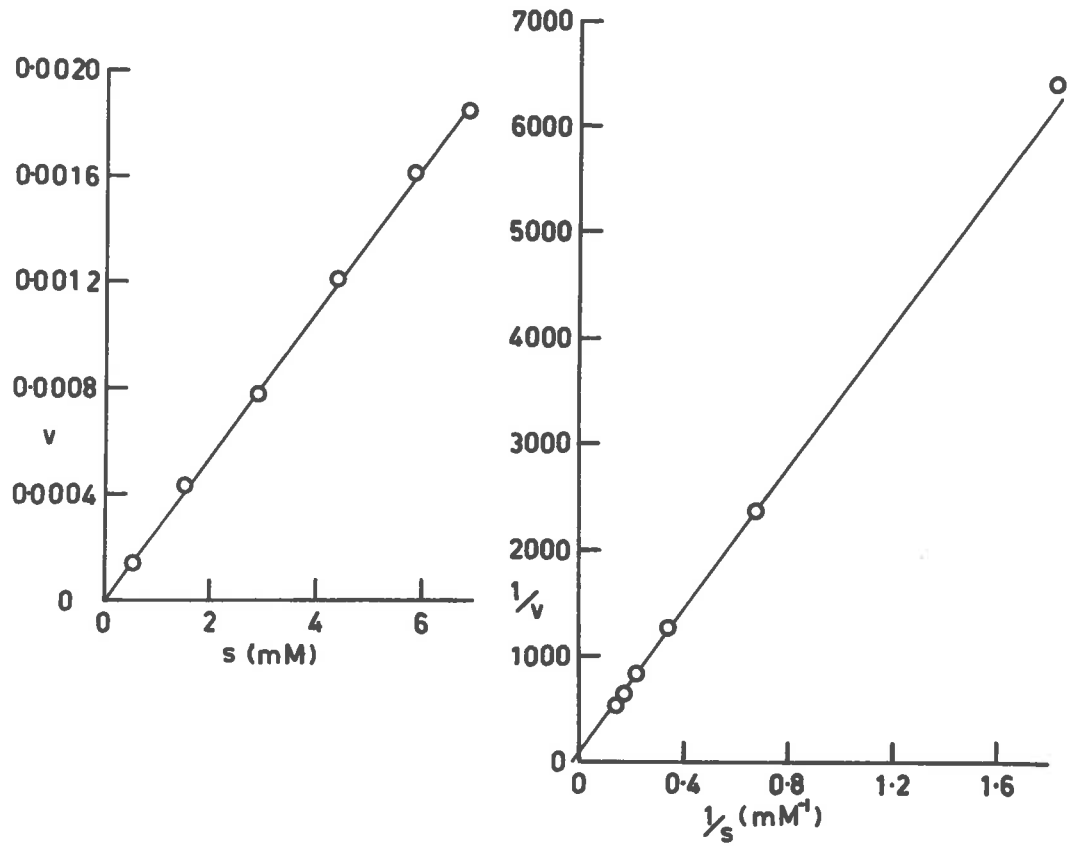


Effect of Mononucleotide Concentration
on the Transferase Reaction :- Thionicotinamide Mononucleotide

Fig.II-6

Fig. II-7

Adenylyl transfer to 3-acetylpyridine mononucleotide



Effect of Mononucleotide Concentration
on the Transferase Reaction :- Acetylpyridine Mononucleotide

Fig. II - 7

study of adenylyltransfer to 3-aldehydepyridine mononucleotide was not attempted because of the very low rates at which this reaction takes place and the extraneous absorption increase discussed above which prevented accurate measurements of dinucleotide formation.

Table II-4

Kinetic constants for adenylyltransfer to NMN and analogues.

	Nucleotide concentration (mM)	µMoles dinucleotide per minute per 107 Y protein
Nicotinamide mononucleotide	0.45	0.0309
	0.90	0.0366
K_m 1.93×10^{-4} M	1.13	0.0373
V_{max} 0.044 µmoles dinucleotide per minute per 107 Y protein	2.26	0.0398
	4.52	0.0432
Thionicotinamide mononucleotide	0.115	0.000253
K_m 8.3×10^{-4} M	0.285	0.000609
	0.575	0.000982
V_{max} 0.0024 µmoles dinucleotide per minute per 107 Y protein	1.15	0.001388
	2.30	0.001590

Table II-4 contd.

3-Acetylpyridine mononucleotide	0.584	0.000156
	1.46	0.000437
$K_m \sim 0.1$ M	2.92	0.000780
$V_{max} >$ thionicotinamide analogue	4.38	0.00122
	5.84	0.00163
	6.95	0.00185

(f) Adenylyl transfer to nicotinic acid mononucleotide

The rates of adenylyl transfer to nicotinic acid mononucleotide and to NMN were compared by estimating the pyrophosphate formed during the reaction (Methods (c)). Many errors are inherent in the method, and generally speaking it is not suitable for accurate kinetic studies. The rates obtained are shown in Table II-5 and the corresponding Lineweaver-Burk plots in Figure II-8. The results indicate that at the concentrations used, transfer to nicotinic acid mononucleotide proceeds about twice as fast as that to NMN. Where NMN was used rates were also checked by determination of NAD with yeast alcohol dehydrogenase (Methods; (a)). A comparison of the two methods in this case shows the lack of precision in rates obtained by pyrophosphate estimation. However, where nicotinic acid mononucleotide was used as substrate, the rates fitted a straight line when plotted according to Lineweaver and Burk, and generally the rates obtained by pyrophosphate determination appeared to have less scatter, possibly because of the higher rates with this substrate compared to

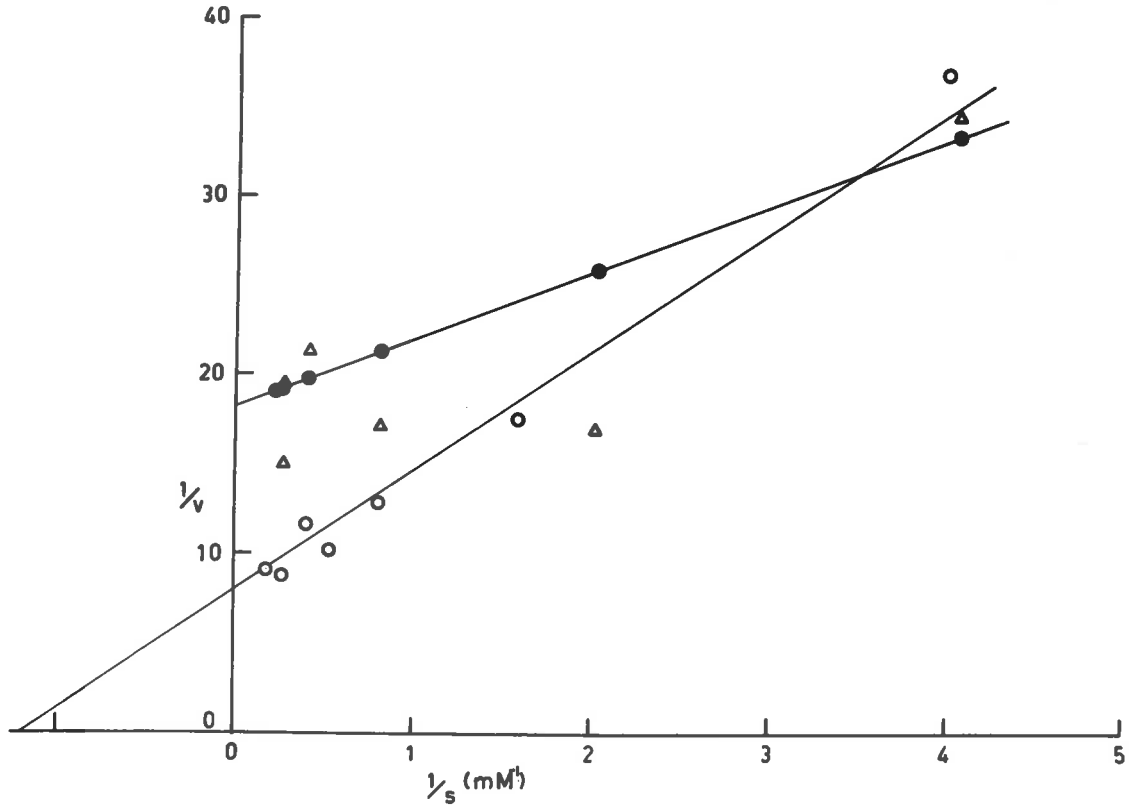
Fig. II-8

Adenylyl transfer to nicotinic acid mononucleotide.

○ transfer to nicotinic acid mononucleotide
(pyrophosphate estimation)

△ transfer to NMN
(pyrophosphate estimation)

● transfer to NMN
(NAD estimation)



Effect of Mononucleotide Concentration
on the Transferase Reaction :- Nicotinic Acid Mononucleotide
and NMN

Fig. II - 8

those for NMN. An analysis of the reciprocal plots indicated that nicotinic acid mononucleotide had a K_m (0.85mM) about four fold greater than that for NMN (0.21mM) under the same conditions. Although this figure for the apparent K_m of nicotinic acid mononucleotide under the above conditions of assay has to be treated with caution in view of the inaccuracies of the method, it does agree reasonably well with conclusions from an analysis of the inhibition by nicotinic acid mononucleotide of adenylyltransfer to NMN (see below).

Table II - 5

A comparison of the rates of adenylyltransfer to NMN and to nicotinic acid mononucleotide.

	Nucleotide concentration (mM)	µMoles pyrophosphate per minute	µMoles NAD per minute
Nicotinamide mononucleotide	0.246	0.0288	0.0298
	0.492	0.0587	0.0388
K_m 0.21mM	1.23	0.0575	0.0470
V_{max} 0.055 µmoles NAD per minute	2.46	0.0470	0.0505
	3.69	0.0665	0.0520
	4.92	0.0527	0.0530
Nicotinic acid mononucleotide	0.25	0.027	-
	0.63	0.057	-
K_m 0.85mM	1.26	0.079	-
V_{max} 0.125 µmoles pyrophosphate per minute	1.89	0.098	-
	2.52	0.086	-
	3.78	0.114	-
	5.04	0.110	-

(g) Inhibition of ATP+NMN adenylyl transfer by NMN analogues

The effect of various analogues of NMN on the rate of adenylyl transfer to NMN was investigated by incubating each analogue with 5mM-ATP, 15mM-$MgCl_2$, 1.10mM-NMN and 100mM-glycylglycine, pH 7.6 in the presence of ATP:NMN adenylyltransferase. The reaction was initiated by the addition of 0.02 ml. of a transferase preparation capable of synthesizing 0.5 µmoles of NAD per minute per mg protein, bringing the total volume to 1.0 ml. After 3 minutes at 37°, the reaction was stopped with 1.5 ml. of 0.5M trichloracetic acid and the NAD synthesized estimated in the usual manner (Methods, (a)). Under these conditions, in the absence of analogues, 0.140 µmoles of NAD were synthesized during the incubation. The ribosides and nucleotides used in this series of experiments, with the exception of nicotinic acid riboside, nicotinic acid mononucleotide and NMN, were supplied by Mr. R. Naylor and had been prepared synthetically. The α-NMN preparation contained,as well as the 5'-phosphate, approximately 20% (molar proportions) of α-nicotinamide riboside-3'-phosphate and 1% β-NMN. This proportion of natural substrate would not have any significant effect at the overall concentrations employed on the rate of NAD synthesis.

As indicated in Table II-6, nicotinic acid mononucleotide was found to be the only effective inhibitor under the conditions used. An analysis of these results, assuming nicotinic acid mononucleotide to be a competitive inhibitor of adenylyltransfer to NMN,

Table II-6

Effect of NMN analogues on ATP:NMN adenylyl transfer.

NMN Analogue	Concentration (mM)	Relative rate
-	-	1.00
Nicotinamide riboside*	1.18	1.04
Nicotinamide riboside*	11.80	0.96
Pyridine mononucleotide*	1.0	1.07
Pyridine mononucleotide*	5.0	1.04
Thionicotinamide mononucleotide*	0.9	1.04
Nicotinic acid riboside	1.89	1.02
Nicotinic acid mononucleotide	0.895	0.825
Nicotinic acid mononucleotide	4.48	0.485
α-Nicotinamide mononucleotide	5.0	0.97
6-Phosphoglucosyl nicotinamide*	2.3	0.99
(*Mixtures of α- and β-anomers)		

was made by plotting the reciprocal of velocity against inhibitor (nicotinic acid mononucleotide) concentration, according to the method of Dixon (1950). In previous work with this enzyme (Atkinson, Jackson & Morton, 1960) it was found that nicotinic acid mononucleotide was a competitive inhibitor (Ki 0.38mM) of adenylyl transfer from ATP (4mM) to NMN; in these experiments NMN had K_m 0.12mM so that K_i (nicotinic acid mononucleotide)/ K_m (NMN)was 3.2. On the basis of NMN having a K_m of 0.2mM in the conditions described in Tables II-5 and II-6

consideration of the slope of the resulting line (figure II-9) indicates that the corresponding ratio of K_i (nicotinic acid mononucleotide)/K_m (NMN) found here is also 3.2, and the K_i is 0.69mM. This value is close to the value of K_m (0.85mM) calculated for nicotinic acid mononucleotide (Table II-5)

(h) Pyridine mononucleotide and adenylyl transfer

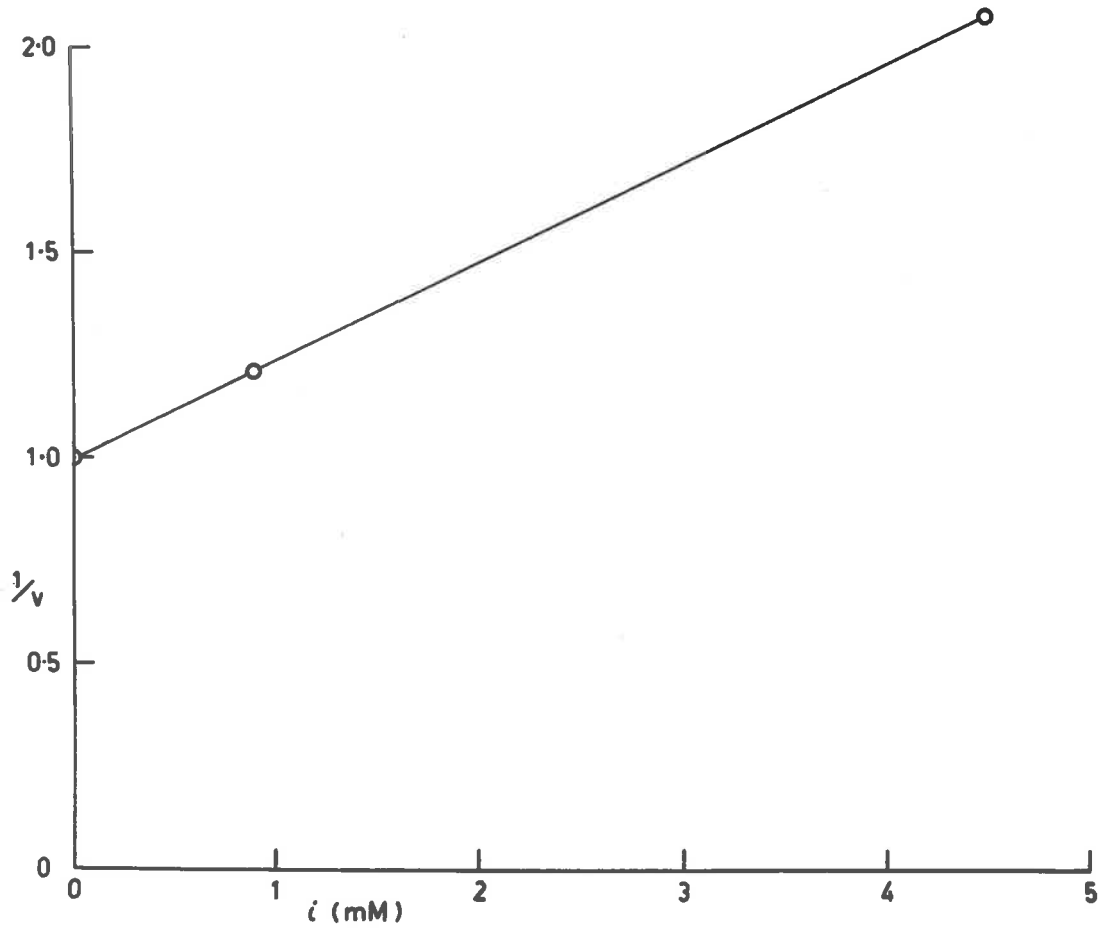
To a reaction mixture containing 1 µmole of ATP, 3.7 µmoles $MgCl_2$, 0.005 units of ATP:NMN adenylyltransferase and 25 µmoles of glycylglycine, pH 7.6, 1 µmole of pyridine mononucleotide was added. The total volume of the reaction mixture was 0.2 ml. After 2.5 hours at 37°, a portion of the mixture (0.025 ml.) was applied to a paper strip (3MM Whatman chromatography paper) and subjected to electrophoresis at pH 4.2 (0.04M-sodium citrate) for 3.25 hours at 400 volts (14 V per cm.) The nucleotides present were then detected on the paper by printing on reflex document paper with ultraviolet light. Under these conditions replacement of the pyridine mononucleotide by NMN (or thionicotinamide mononucleotide) gave rise to a new nucleotide component which moved with the same mobility on electrophoresis as NAD (or thionicotinamide adenine dinucleotide). However, no dinucleotide component was detected when pyridine mononucleotide was included in the reaction mixture, suggesting that the transferase will not catalyse adenylyl transfer to this analogue.

(i) Pyrophosphorolysis of NAD analogues.

The pyrophosphorolysis of thionicotinamide adenine dinucleotide, 3-acetylpyridine adenine dinucleotide and 3-pyridinealdehyde adenine dinucleotide was demonstrated by incubating 1 µmole of each of these

Fig. II-9

Inhibition of ATP:NMN adenylyl transfer by nicotinic acid mononucleotide



Inhibition of ATP:NMN Adenylyl Transfer
by Nicotinic Acid Mononucleotide

Fig. II - 9

analogues with 20 μmoles of pyrophosphate, 3.7 μmoles $MgCl_2$, 25 μmoles of glycylglycine, pH 7.6 and 0.005 units of ATP:NMN adenylyltransferase (total volume 0.2 ml.) After 2.5 hours at 37°, the products were subjected to electrophoresis as above. As with NAD, these dinucleotide analogues gave rise to new nucleotide components with the same mobility as ATP and NMN after electrophoresis, thus establishing that the transferase catalyses a freely reversible reaction with these analogues, just as it does with the natural substrate.

D. Discussion

In previous work (Jackson, 1960; Atkinson et al., 1961) a sensitive modification of the routine transferase assay was used to give reliable initial rates for the calculation of K_m for NMN. With the NMN analogues the high absorbances of some of the compounds at 340 mμ and the small quantities of the analogues available have prevented the use of the modified assay. K_m for NMN in the experiments described here (cf. Table II-5) is about twice the value found with the more sensitive assay; this discrepancy may also be partly due to the use of a different buffer system and reaction temperature. Though a similar discrepancy may exist for the K_m values of the analogues studied here the values obtained for relative V_{max} and K_m provide a useful indication of the structural factors required for dinucleotide formation with this enzyme.

Table II-7 summarizes the kinetic data obtained for NMN and its analogues, in which the side chain at the 3 position of the

pyridine ring has been altered.

Table II-7

Specificity of ATP:NMN adenylyltransferase - NMN analogues

Nucleotide	3-Side chain	Apparent K_m (mM)	Apparent V_{max} (relative)
Nicotinamide mononucleotide	$-\overset{O}{\overset{\|}{C}}-NH_2$	0.2	100
Nicotinic acid mononucleotide	$-\overset{O}{\overset{\|}{C}}-O^-$	0.85	ca. 200
Thionicotinamide mononucleotide	$-\overset{S}{\overset{\|}{C}}-NH_2$	0.83	5.5
3-Acetylpyridine mononucleotide	$-\overset{O}{\overset{\|}{C}}-CH_3$	ca. 100	5.5
3-Aldehydepyridine mononucleotide	$-\overset{O}{\overset{\|}{C}}-H$	-	slight reaction
Pyridine mononucleotide	-H	-	no reaction

It seems that a substituent in the pyridinium ring is essential for activity in the reaction catalysed by the transferase, since pyridine mononucleotide is not a substrate. In addition, any alteration in the 3-carboxamide group of NMN results in a less favourable (higher) K_m. It is noteworthy that the two substrates with highest V_{max} and lowest K_m (NMN and nicotinic acid mononucleotide) each have a substituent with two relatively polar groups. The groups concerned are capable of participating in hydrogen bond formation, the amino group being able to act as a proton donor or acceptor and the carbonyl and dissociated hydroxyl groups as acceptors (Bernal, 1959). It may be

that NMN, the substrate with the most favourable K_m, is bound to the enzyme through its amide, which could form one or two hydrogen bonds to a group in the active centre.

From an examination of molecular models it is evident that β-NMN can take up a compact conformation with a hydrogen bond between the amino group of the amide and an oxygen of the phosphate. This conformation is probably favoured by the close approach which it permits between the anionic phosphate and the cationic nitrogen atom of the pyridine ring. Neither the hydrogen bond nor the ionic interaction is likely in nicotinic acid mononucleotide, which probably has a different shape in solution. Reduction in polarity of either of the two potential hydrogen bonding groups in the amide substituents of NMN leads to a higher K_m, perhaps reflecting a lowering in affinity for the enzyme. Thus when the oxygen atom of the carbonyl group is substituted by a sulphur atom, or when the amino group is replaced by a methyl group, the K_m is raised (Table II-7). Both these groups are known to have less tendency to form hydrogen bonds, particularly in the case of themethyl moiety. The rate of reaction is reduced still further by substituting a hydrogen atom (as in 3-aldehyde pyridine mononucleotide) for the methyl group. The methyl group might still interact weakly by van der Waals' forces with structures in the active centre.

In a previous investigation (Atkinson et al., 1961) it was found that a group with an apparent pK of 9.97 ± 0.07 was involved in the binding of NMN. As there is no corresponding dissociation in the NMN it is probably that of a group in the enzyme (e.g.

ε-ammonium of a lysyl residue or -OH of a tyrosyl residue) or in the enzyme-ATP-magnesium complex. It has not been possible to identify this binding group, but hydrogen bond formation between the amide group of the nucleotide and a tyrosyl residue of the enzyme might be involved in the enzyme-substrate interaction.

Nicotinic acid mononucleotide was shown to be a substrate for ATP:NMN adenylyltransferase by Preiss and Handler (1958), who compared the rate of adenylyl transfer to NMN and to nicotinic acid mononucleotide at various stages in the partial purification of the enzyme from pig liver acetone powder. It was found that adenylyl transfer proceeded at approximately equal rates with 0.4mM nicotinic acid mononucleotide and 2mM NMN throughout the preparation. It seemed that the transfer took place at a higher rate with the acid analogue than with NMN (Imsande and Handler, 1961). The equality of rates at 0.4mM and 2mM concentrations of the respective nucleotides is consistent with the relative values of K_m and V_{max} quoted here for the two analogues (Table II-5).

The higher V_{max} for nicotinic acid mononucleotide may be an expression of the greater overall nucleophilic character of this analogue, compared to NMN, due to the carboxylic group which is dissociated at a physiological pH (Lamborg et al., 1958). As discussed above, in NMN, there may be a tendency for the 5-phosphoryl group to form an internal salt with the postively charged quaternary pyridinium nitrogen atom. With nicotinic acid mononucleotide on the other hand, the phosphoryl group may not show such a tendency, due to

the proximity of the negatively charged carboxylic group to the quaternary nitrogen, leaving the phosphoryl group greater freedom in carrying out a nucleophilic attack on the 2-phosphorus atom of ATP.

The only other NMN analogue which has been shown to be a substrate for the transferase is reduced NMN. This analogue reacts at about 30% the rate of NMN (Kornberg, 1950), indicating that the positive charge on the quaternary nitrogen is not essential for binding. The relatively small decrease in rate obtained by substituting the reduced NMN for oxidized NMN may be the result of a slight change in overall shape of the nucleotide on reduction; the hydrogen bonding potentialities of the amide group would also be changed by this reduction.

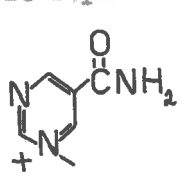
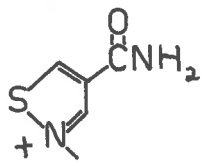
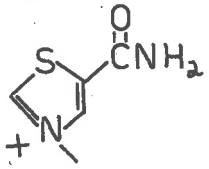
Kornberg (1950) also showed that nicotinamide riboside is not a substrate. The present work indicates that at a concentration of 11 times that of NMN (present at 1mM) the riboside causes less than 5% inhibition of adenylyl transfer (Table II-6). Hence although the substituent on the pyridinium ring is essential for binding, other groups are also needed, since the riboside is a relatively ineffective inhibitor. Presumably the phosphoryl group of NMN is also bound in some way, perhaps by electrostatic forces. Pyridine mononucleotide was not found to be inhibitory at the concentrations used - five times that of NMN. These two experiments, together with other inhibitory studies (Table II-6) indicate the importance of a 'multiple attachment' in the binding of NMN to the transferase.

The steric specificity of the interaction is evident from the fact that the α-5'-phosphoribofuranosyl-, (α + β)- 6'-phosphoglucopyran-

osyl- and 2'-phosphohydroxyethyl- analogues of NMN are neither substrates of the transferase nor effective competitive inhibitors of adenylyl transfer to NMN. (The last of these analogues was prepared and tested by Dr. M. R. Atkinson).

From the results obtained so far it seems likely that effective competitive inhibition of this adenylyl transfer might be obtained with NMN analogues having the following structural features:-

i. a hydrogen bonding group in the position <u>meta</u> to the quaternary nitrogen (e.g. $-SO_2NH_2$, -CONHMe, -CONHOH);

ii. isosteric replacements of atoms in the pyridinium ring (e.g.  ,  , );

iii. slight modifications of the ribofuranosyl portion (eg. β-5'-phosphoxylofuranosyl or β-5'-phosphoarabinofuranosyl derivatives of nicotinamide)

iv. replacement of the phosphate by groups with a similar charge at physiological pH values (eg. $-CH_2-PO_3^{2-}$, $-CH_2AsO_3^{2-}$).

III

Specificity of ATP:NMN adenylyltransferase with respect to ATP analogues.

This section describes experiments in which a number of nucleoside 5'-triphosphates were tested as nucleotidyl donors with the transferase and as inhibitors of adenylyl transfer from ATP to NMN.

A. Materials

Nucleotides. NAD, ATP, deoxyATP (lot number 128-693-1), ITP from muscle, lot number 126-640), GTP (lot number 31B-732-3), UTP and CTP were obtained from Sigma Chemical Co. Pabst Laboratories supplied 8-azaguanosine-5'-triphosphate (lot number X30351), deoxy GTP, nicotinamide hypoxanthine dinucleotide, 3-acetyl-pyridine hypoxanthine dinucleotide and 3-pyridinealdehyde hypoxanthine dinucleotide.

6-Mercapto-purine riboside was purchased from Sigma Chemical Co. (lot number M 110B-73) and Californian Corporation for Biochemical Research, Los Angeles (lot number 441011).

ATP:NMN adenylyltransferase. A preparation capable of synthesizing 0.54 μmoles of NAD per minute per mg. of protein was used, unless otherwise stated. This was prepared as described in Table I-7. For kinetic studies it was dialysed against 0.01M-sodium phosphate, pH 7.5, and diluted with ice-cold water before use.

B. Methods

Determination of the rate of nucleotidyl transfer from ATP and analogues to NMN

(a) Non-coupled assay

The reaction mixture contained 5mM-NMN, 100mM-glycylglycine, pH 7.6, $MgCl_2$, and a nucleoside triphosphate. In view of the dependence of rates of nucleotidyl transfer on the ratio

of the concentrations of nucleoside triphosphate to metal ion (see Section IV), total $MgCl_2$ concentration was maintained at twice that of the nucleoside triphosphate. Whenever the concentration of the latter was varied, $MgCl_2$ was varied in the same ratio. The reaction was initiated by the addition of ATP:NMN adenylyltransferase, bringing the total volume to 1.0 ml. After an appropriate time interval at 37°, the reaction was stopped by adding 1.5 ml. of cold 0.5M-trichloracetic acid solution, and the resultant mixture was centrifuged. 2.0 Millilitres of the supernatant was added to a spectrophotometer cuvette, of 4 cm. light path, followed by 3.0 ml. of 0.835M-ethanol-0.417M-glycine - 0.416M-sodium hydroxide. After the solution had been mixed the amount of NAD (or analogue) present was estimated by recording the optical density of the solution at 340 mμ before and after the addition of 200 γ of yeast alcohol dehydrogenase (0.025 ml.). A millimolar extinction coefficient of 6.22 at 340 mμ was assumed for those NAD analogues in which the adenine moiety was replaced by hypoxanthine or guanine (c.f. Pullman et.al., 1952). Although under these conditions the reduction of NAD is complete in approximately one minute, the reduction of nicotinamide hypoxanthine dinucleotide (and nicotinamide guanine dinucleotide) is not complete until 90 minutes after the addition of dehydrogenase. This is in keeping with the low rate of reduction and the high K_m of both the hypoxanthine analogue (Pullman et al., 1952; van Eys et al., 1958) and the guanine analogue (see below) with yeast alcohol dehydrogenase.

(b) Coupled assay

Nucleotidyl transfer in this case was carried out in a spectrophotometer cuvette of 1 cm. light path. It contained 200 μmoles of glycylglycine, 500 μmoles of ethanol, 4 μmoles of NMN, 16 μmoles of $MgCl_2$, 82Y of yeast alcohol dehydrogenase and a nucleoside triphosphate in a total volume of 2.5 ml. and at pH 8.0. The reaction was started by the addition of pig liver ATP:NMN adenylyltransferase and its progress followed by measuring the increase in absorption at 340 mμ due to the formation of reduced dinucleotide. A recording DK2 Beckman spectrophotometer was used for this purpose. The rate of transfer was calculated from the rate of formation of reduced dinucleotide, using a millimolar extinction coefficient of 6.22 at 340 mμ.

Qualitative aspects of specificity - examination by paper electrophoresis

For dinucleotide synthesis the reaction mixture contained 25 μmoles of glycylglycine, pH 7.6, 3.7 μmoles of $MgCl_2$, 1 μmole of NMN, 1 μmole of nucleoside triphosphate and 0.005 units of ATP:NMN adenylyltransferase, in a total volume of 0.2 ml. The reaction, if any, was initiated by the addition of transferase, and allowed to proceed for 2.5 hours at 37°. After this time interval a portion of the mixture (0.02 to 0.05 ml.) was applied to a strip of chromatography paper (Whatman 3 MM) and subjected to electrophoresis at pH 4.2 (0.04M sodium citrate) for 3.5 hours at 400 V (approximately 14 V. per cm.). The separated nucleotides were detected on the paper strip (after drying), by printing on reflex document paper with ultra violet light (see Atkinson et al., 1961b). Quaternary nicotinamide compounds were detected by

their fluorescence after exposure to an atmosphere of methylethyl ketone-ammonia (Carpenter and Kodicek, 1950).

The pyrophosphorolysis of NAD analogues was detected by incubating the dinucleotide (1 μmole) with 25 μmoles of glycylglycine, pH 7.5, 3.7 μmoles of $MgCl_2$, 20 μmoles of pyrophosphate and 0.005 units of the transferase, contained in a total volume of 0.2 ml. The reaction was started by the addition of transferase, and after 2.5 hours at 37° a portion of the reaction mixture was analysed for its nucleotide content by paper electrophoresis as above.

Preparation of 6-mercaptopurine nucleotides

6-Mercaptopurine riboside-5'-phosphate was prepared from the nucleoside in a 25% yield by the method of Roy et al., (1961). The compound migrated as one component on chromatography in 95% ethanol-1M-ammonium acetate, pH 7.5 (7:3;v/v) and in 1% ammonium sulphate-isopropanol (1:2;v/v). Paper electrophoresis showed the product to have the same mobility as IMP at pH 4.2, and in addition the nucleotide on paper gave a positive test for reducing sulphur compounds (Toennies and Kolb, 1951). The ultraviolet spectra of the compound in 0.1M-sodium phosphate at several pH values were in accord with those reported for the corresponding riboside by Fox et al., (1958). The spectrum showed a dependence on pH (5.7 to 8.0) and an isosbestic point at 311 mμ was apparent.

The 6-mercaptopurine riboside-5'-phosphate was converted into the nucleoside triphosphate by treatment with orthophosphate and dicyclohexylcarbodiimide in dry pyridine (Roy et al., 1961). After

removal of pyridine and carbodiimide, the nucleotide products of the reaction were adsorbed to charcoal (Norite A), the charcoal was washed with water, and the nucleotides were eluted with 50% ethanol-2% ammonia solution. This step removes inorganic phosphates and polyphosphates from the mixture. The eluate, after removal of ethanol and ammonia in vacuo, apparently contained 6-mercaptopurine riboside -5'-monophosphate, -5'-diphosphate and -5'-triphosphate. Paper electrophoresis of the mixture (preparation A) showed three components which absorbed light at 254 mμ and had the same mobilities at pH 4.2 as inosine -5'-monophosphate, -5'-diphosphate and -5'-triphosphate. Each component gave a positive test for reducing sulphur compounds (Toennies and Kolb, 1951). Ion exchange chromatography of preparation A on ECTEOLA cellulose (Roy et al., 1961) gave four fractions, the last of which was subsequently shown by enzymic assay to contain 6-mercaptopurine riboside-5' triphosphate (preparation B) (Atkinson et al., 1962). The yield of this nucleotide was too low to permit characterization.

C. Results

(a) Nucleotidyltransfer from ATP analogues to NMN

The following nucleoside triphosphates, when incubated with NMN, $MgCl_2$ and ATP:NMN adenylyltransferase, were found to give rise to a nucleotide component which had the same mobility as NAD or the corresponding dinucleotide analogue on paper electrophoresis: ATP, ITP, GTP deoxy ATP and 6-mercaptopurine riboside-5'-triphosphate (preparation A). The new component in each case was shown to

contain a quaternary nicotinamide moiety. The results suggest that nucleotidyl transfer had taken place to give a nicotinamide adenine dinucleotide analogue. ATP and deoxy ATP appeared to react much faster than the other three nucleoside triphosphates. The new compound formed with the 6-mercaptopurine derivative gave a positive reaction for -SH.

No new nucleotide component was observed, after electrophoresis, by photographing the paper strip in ultraviolet light, when analysing the reaction mixtures containing 8 aza GTP or deoxy GTP. However, in both these cases the more sensitive technique of Carpenter and Kodicek (1950) showed that a compound containing a quaternary nicotinamide moiety was present in the region expected for a NAD analogue. It is thus probable that both these nucleoside triphosphates can act as a nucleotidyl donor to NMN, but only at an extremely low rate.

No reaction was apparent when UTP or CTP was substituted for ATP.

(b) Pyrophosphorolysis of NAD analogues

Using the techniques described in the Methods section, nicotinamide hypoxanthine dinucleotide in the presence of pyrophosphate and ATP:NMN adenylyltransferase was shown to give rise to two products which absorbed ultraviolet light and had the same mobilities as NMN and ITP on paper electrophoresis. In addition the component with the same mobility as NMN fluoresced when treated according to the method of Carpenter and Kodicek (1950). Hence the reversibility of the

transfer, in which the adenine moiety of the substrates is replaced by hypoxanthine is established.

The substitution of 3-acetylpyridine hypoxanthine dinucleotide and 3-pyridinealdehyde hypoxanthine dinucleotide for nicotinamide hypoxanthine dinucleotide in the above reaction did not give rise to any new nucleotide components. These two dinucleotides do not appear to have any reactivity in the system.

(c) Preparation and properties of nicotinamide guanine dinucleotide

GTP (75 mg.; 124 μmoles) was incubated at 37° with 100 μmoles of NMN, 2.5 millimoles of glycylglycine, 300 μmoles of $MgCl_2$ and 5 units of ATP:NMN adenylyltransferase. The total volume of the reaction mixture was 10 ml. and the pH 7.7. The transferase used was capable of synthesizing 2.7 μmoles of NAD per minute per mg. protein. After 4 hours, enough inorganic pyrophosphatase (from yeast) to hydrolyse 1 μmole per minute of pyrophosphate was added and the reaction was allowed to proceed for a further 5 hours. The mixture was stored frozen overnight. After thawing, it was acidified with perchloric acid and the precipitated protein removed by centrifugation. The supernatant was titrated to pH 7.0 with 4N-potassium hydroxide solution. After 24 hours at 2°, the precipitated potassium perchlorate was filtered off and the filtrate was applied to a column of Dowex-2 formate (200-400 mesh). The resin was first washed with water and then subjected to gradient elution with an increasing concentration of formic acid. 3 Millilitre fractions were collected

on an automatic fraction collector. NMN was eluted with water, and what proved to be nicotinamide guanine dinucleotide with 1.0 to 1.5N-formic acid. The fractions containing this compound were combined and freeze dried. The residue was dissolved in 0.01N-HCl.

The properties of the compound are consistent with those of a dinucleotide of nicotinamide and guanine. The spectra of the compound in M-KCN and 0.1N-HCl solutions is shown in figure III-1. In M-KCN it has a maximum absorption at 266 mμ and at 325 mμ. On the basis of the millimolar extinction coefficient at 325 mμ in cyanide being the same as that of NAD (6.3; see Colowick et al., 1951), then 10 μmoles of the compound were obtained from the enzymic reaction (a yield of 8% from GTP). The new dinucleotide migrated as one component on ascending chromatographyin isobutyric acid - water - concentrated ammonia (33:66:1; vols.). In this system, where NAD was found to have an R_f of 0.45, the analogue had an R_f of 0.32. On paper electrophoresis at pH 4.2 (0.04M-sodium citrate; 10V per cm. for 3:5 hours) the compound again moved as one component with a mobility between that of NAD and AMP.

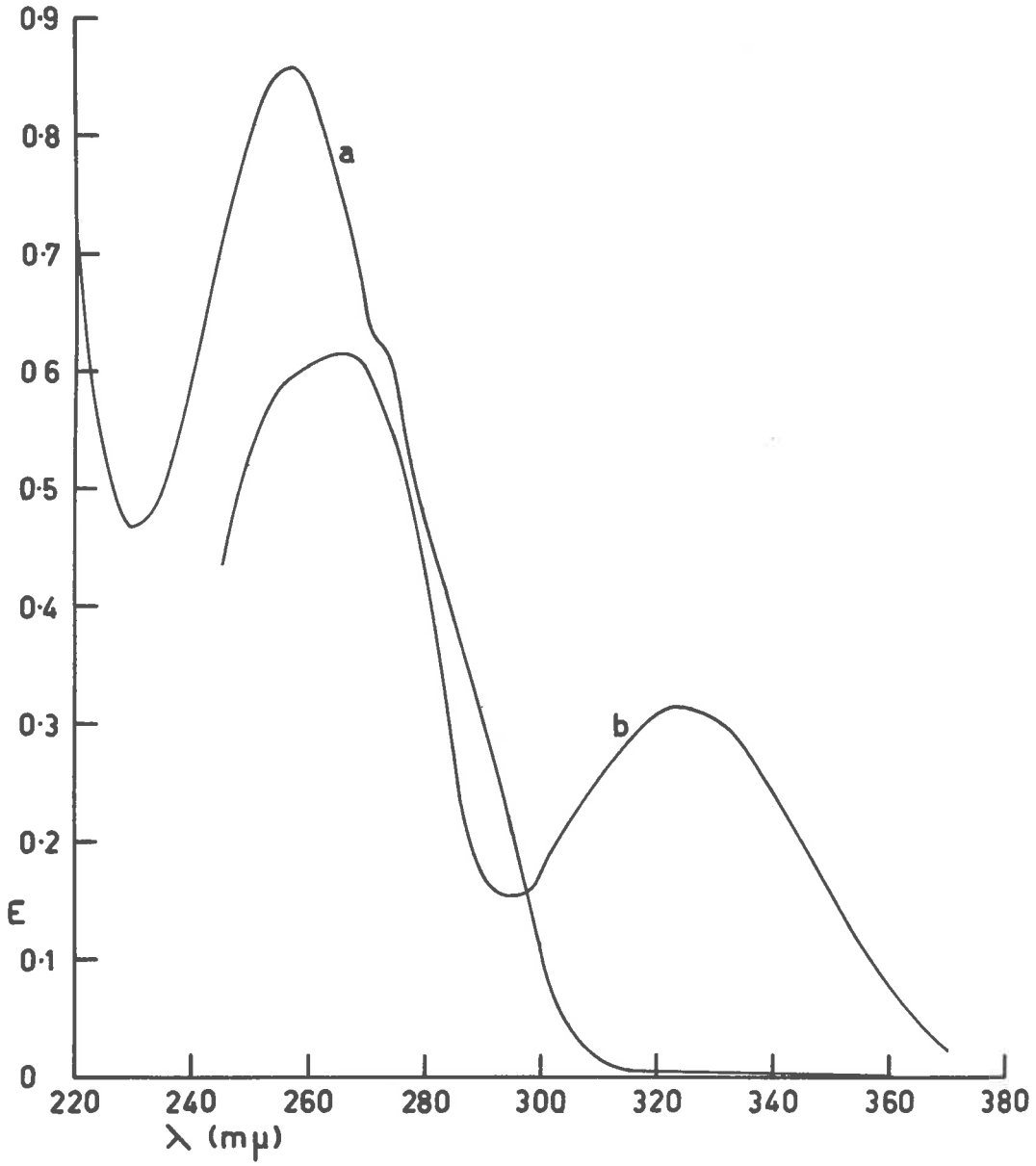
When the compound was hydrolysed in 50% formic acid at 180° in a sealed tube, chromatography of the product gave two ultraviolet absorbing components, one of which had the same R_f as guanine and the other the same as nicotinic acid. The former component was eluted from the paper and gave an ultraviolet absorption spectrum identical with that of guanine which had been chromatographed and eluted alongside the hydrolysate. NAD, under the same conditions of hydrolysis, gave adenine and nicotinic acid. The hydrolysis of these dinucleotides,

Fig. III-1

U.V. spectrum of nicotinamide guanine dinucleotide

(a) in $^{N}/_{10}$ HCl

(b) in M KCN



U.V. Spectrum of Nicotinamide Guanine Dinucleotide

Fig. III - 1

and the subsequent chromatography of the products, was carried out using the method of Wyatt (1951). A summary of the properties of nicotinamide guanine dinucleotide is given in Table III-1.

Table III-1

A comparison of the properties of NAD and nicotinamide guanine dinucleotide.

Property	Nicotinamide adenine dinucleotide	Nicotinamide guanine dinucleotide
R_f on chromatography in isobutyric acid - water - ammonia (33.66:1; vols)	0.45	0.32
Treatment with methylethylketone-ammonia (Carpenter and Kodicek, 1950)	fluorescent	fluorescent
Heterocyclic compounds liberated by hydrolysis	adenine and nicotinic acid	guanine and nicotinic acid
Absorption spectrum in 0.1N-HCl	λ_{max} 260 mμ	λ_{max} 257 mμ ($m\varepsilon$, 17.2) inflexions: 273 and 288 mμ
Absorption spectrum in M-KCN	λ_{max} 260 mμ and 325 mμ E_{260}/E_{325} = 2.39	λ_{max} 266 mμ and 325 mμ E_{266}/E_{325} = 1.96
Reaction with yeast alcohol dehydrogenase and ethanol (pH 10)	K_m 1.6×10^{-4} M	$K_m > 2 \times 10^{-3}$ M V_{max} about 2% of V_{max} with NAD

Nicotinamide guanine dinucleotide can be reduced by ethanol and yeast alcohol dehydrogenase. Like NAD and nicotinamide hypoxanthine dinucleotide (Siegal et al., 1959; Pullman et al., 1952), the reduced guanine analogue has an absorption maximum near 340 mμ. The effect of the concentration of nicotinamide guanine dinucleotide on its rate of reduction by yeast alcohol dehydrogenase in 0.1M-'Tris', pH 10.0 - 0.05M-ethanol was briefly studied. The reaction was started by the addition of dehydrogenase to the reaction mixture in a spectrophotometer cuvette of 0.5 cm. light path. The inital rate was determined as the optical density change at 340 mμ over the first 30 seconds of the reduction. Figure III-2 shows the Lineweaver-Burk plot of the results for analogue reduction compared to that for NAD reduction. The latter was obtained in the same way except that a spectrophotometer cuvette of 4 cm. light path and a different concentration of dehydrogenase was used. Under these conditions NAD showed a K_m of 1.6×10^{-4}M and nicotinamide guanine dinucleotide 2×10^{-3}M or higher. The relative concentrations of dehydrogenase used to obtain these two results was not determined, so that the relative magnitude of V_{max} for the two analogues cannot be ascertained from this experiment. However, earlier experiments showed the rate of reduction of nicotinamide guanine dinucleotide to be about 2% of that with NAD, at a dinucleotide concentration of 0.5mM.

(d) Kinetics of nucleotidyl transfer from ITP to NMN

The rate of inosinyl transfer from ITP to NMN was compared

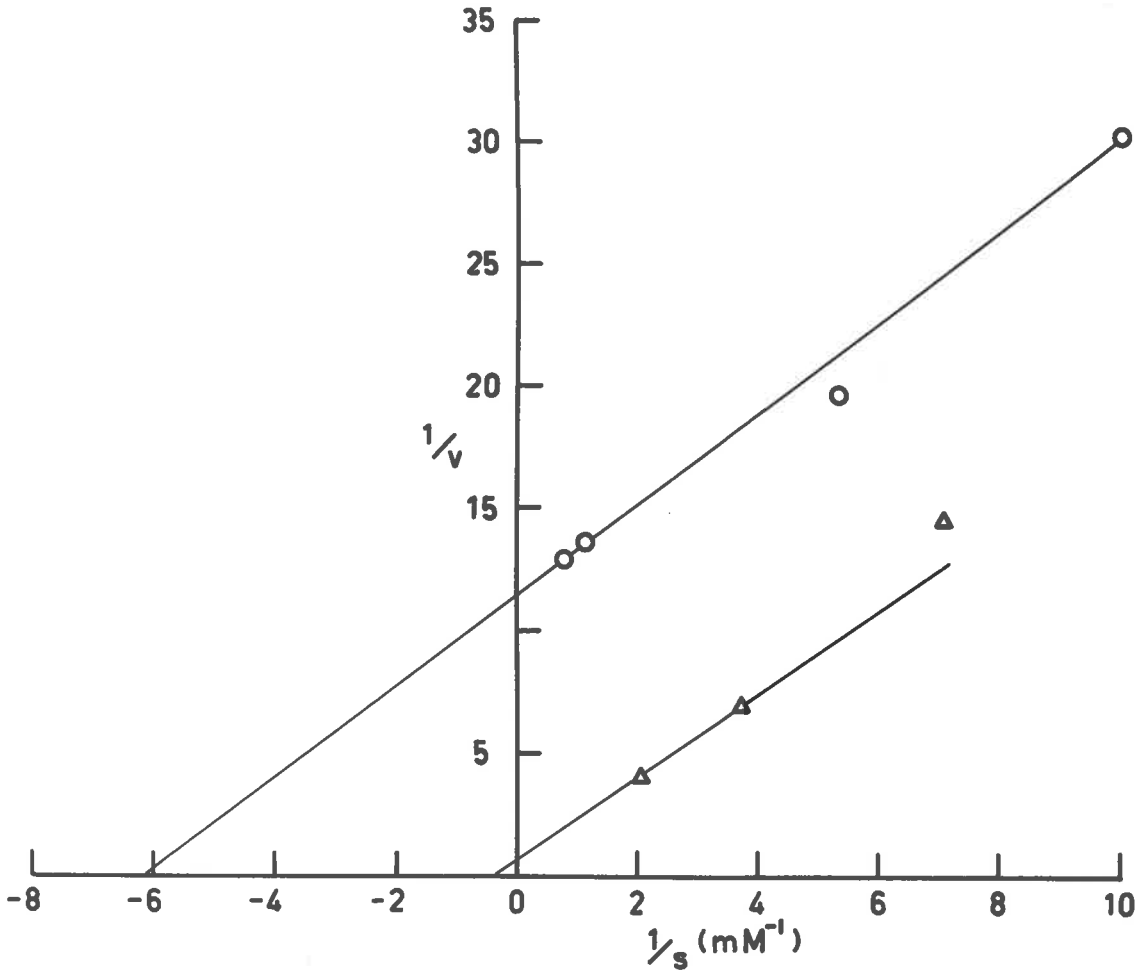
Fig. III-2

Reduction of NAD and nicotinamide guanine dinucleotide

by yeast alcohol dehydrogenase

○ NAD

△ Nicotinamide guanine dinucleotide



Reduction of NAD and Nicotinamide Guanine Dinucleotide
by Yeast Alcohol Dehydrogenase

Fig.III-2

to adenylyl transfer from ATP by means of the direct assay (Methods(a)). For the former triphosphate the substrates were incubated with an ATP:NMN adenylyltransferase preparation containing 500 γ of protein for 30 minutes at 37°. Adenylyl transfer was carried out with 83.3 γ of protein over a period of 5 minutes at the same temperature. The rate of transfer in both cases was calculated as μmoles of NAD or analogue synthesized per minute per 500 γ of protein. Table III-2 shows the results obtained, and the corresponding Lineweaver-Burk plots are given in figure III-3.

Thus ITP has an apparent K_m which is 4.25 fold greater than that for ATP, while the apparent maximal velocity with the analogue is only $^1/_{133}$ of that with the natural substrate (Table III-2). If K_m obtained in this way is a measure of the affinity of the substrate for the enzyme then ITP should be a relatively effective inhibitor of adenylyl transfer from ATP to NMN, with a K_i similar in value to that for the K_m of ITP.

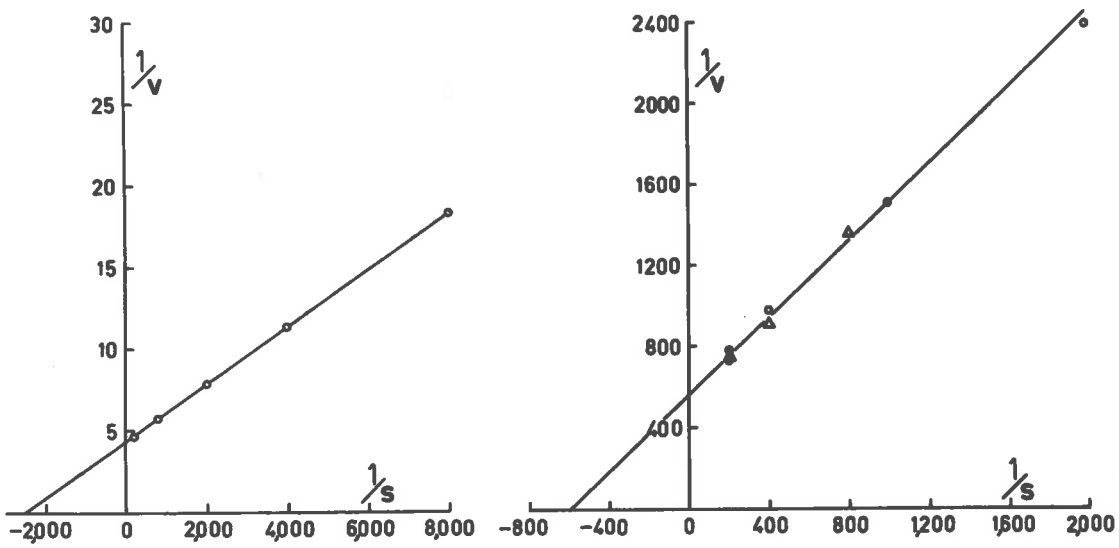
For the transferase acting on ATP and ITP together, the rate of total dinucleotide formation is given by

$$v = v_{ATP} + v_{ITP} = \frac{V_{ATP}}{1 + \frac{K_{ATP}}{s_{ATP}}\left(1 + \frac{s_{ITP}}{K_{ITP}}\right)} + \frac{V_{ITP}}{1 + \frac{K_{ITP}}{s_{ITP}}\left(1 + \frac{s_{ATP}}{K_{ATP}}\right)}$$

(Dixon and Webb, 1958, equation IV-65, p.92), where V_{ATP} and V_{ITP} are maximal rates attained in the presence of either ATP or ITP, s is the nucleoside triphosphate concentration giving the rate of dinucleotide formation, v, and K_{ATP} and K_{ITP} are the K_m values for

Fig. III-3

Nucleotidyl transfer to NMN from ATP and from ITP



ATP

$K_m = 4 \times 10^{-4} M$

$V_{max} = 0{\cdot}233$

ITP

$K_m = 1{\cdot}7 \times 10^{-3} M$

$V_{max} = 0{\cdot}00175$

Fig. III - 3

Table III-2

A comparison of ATP and ITP as substrates for ATP:NMN adenylyltransferase.

Nucleoside triphosphate	Concentration (mM)	μMoles NAD or analogue per minute per 500 Y protein
ATP	0.025	0.0115
	0.050	0.0226
	0.125	0.0548
K_m 4×10^{-4} M	0.250	0.0885
	0.50	0.1275
V_{max} 0.233 μmoles NAD	1.25	0.1728
per minute	5.0	0.212
per 500 Y protein	10.0	0.229
	12.5	0.233
ITP	0.5	0.000419
	1.0	0.000662
	1.25	0.000737
K_m 1.7×10^{-3} M	2.5	0.00102
	2.5	0.00110
V_{max} 0.00175 μmoles	5.0	0.00136
NAD analogue	5.0	0.00127
per minute	5.0	0.00133
per 500 Y protein		

ATP and for ITP respectively. Since V_{ITP} is much smaller in magnitude than V_{ATP} the last term in the above equation becomes negligible and the equation takes the form

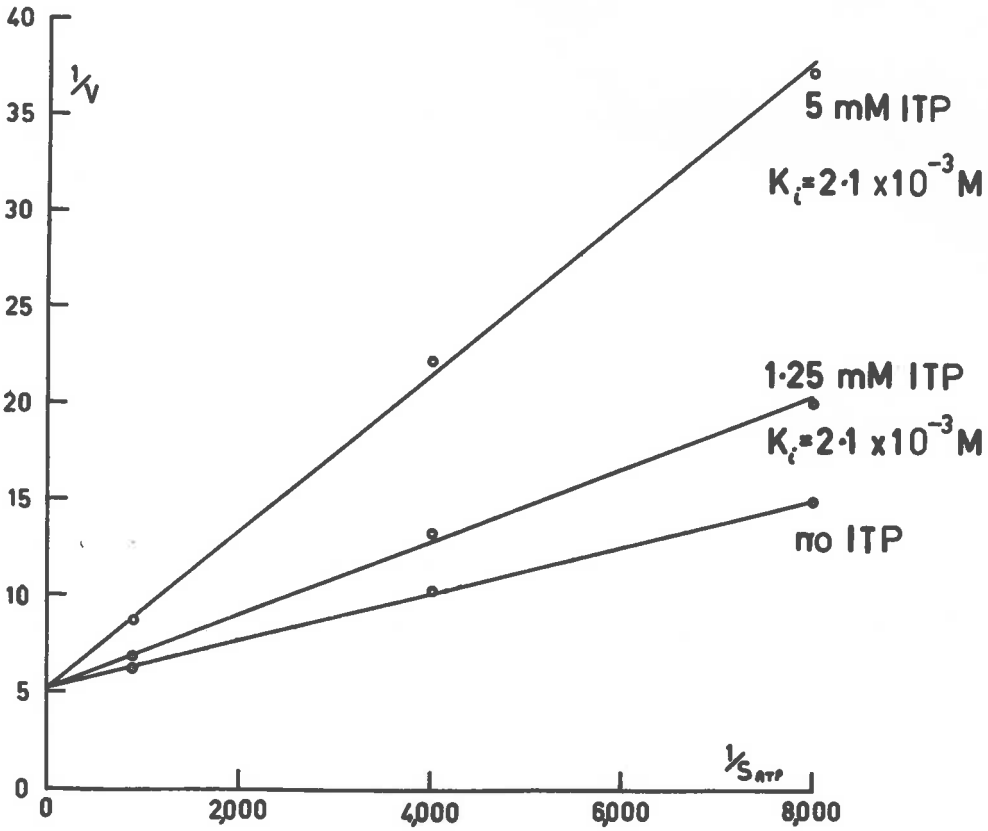
$$v = \frac{V_{ATP}}{1 + \frac{K_{ATP}}{s_{ATP}}(1 + \frac{s_{ITP}}{K_{ITP}})}$$

This is of the same form for the equation describing competitive inhibition (Dixon and Webb, 1958, equation IV-214, p.173), and predicts that ITP is a competitive inhibitor of adenylyl transfer with a K_i equal in value to the K_m for ITP participating in inosinyl transfer.

To test this prediction, the inhibition of adenylyl transfer by ITP was observed over a tenfold range of ATP concentration (0.125 to 1.25 mM) at 1.25 and 5.0 mM-ITP. Again the techniques described in Methods (a) were used to measure the rate of formation of NAD. Under the conditions used, no measurable quantity of nicotinamide hypoxanthine dinucleotide was formed. The reaction was initiated by adding the transferase preparation containing 83.3 γ of protein to the reaction mixture, and was allowed to proceed for 5 minutes at 37°. The rate of adenylyl transfer in the presence of ITP is shown in Table III-3. A Lineweaver-Burk plot (figure III-4) indicated competitive inhibition by ITP, with a K_i of 2.1 mM. This is in reasonable agreement with the K_m obtained above for ITP (1.7 mM).

Fig. III-4

Inhibition of ATP:NMN adenylyl transfer by ITP.



INHIBITION OF ATP : NMN ADENYLYLTRANSFERASE BY ITP

Fig. III - 4

Table III-3

Inhibition of adenylyl transfer to NMN by ITP.

ATP concentration (mM)	ITP concentration (mM)	µMoles NAD per minute per 500 γ protein
0.125	0	0.0669
0.25	0	0.0965
1.25	0	0.1618
1.25	0	0.1592
1.25	0	0.1630
0.125	1.25	0.0498
0.25	1.25	0.0745
1.25	1.25	0.1462
0.125	5.0	0.0268
0.25	5.0	0.0449
1.25	5.0	0.1141

(e) Kinetics of nucleotidyl transfer from GTP to NMN

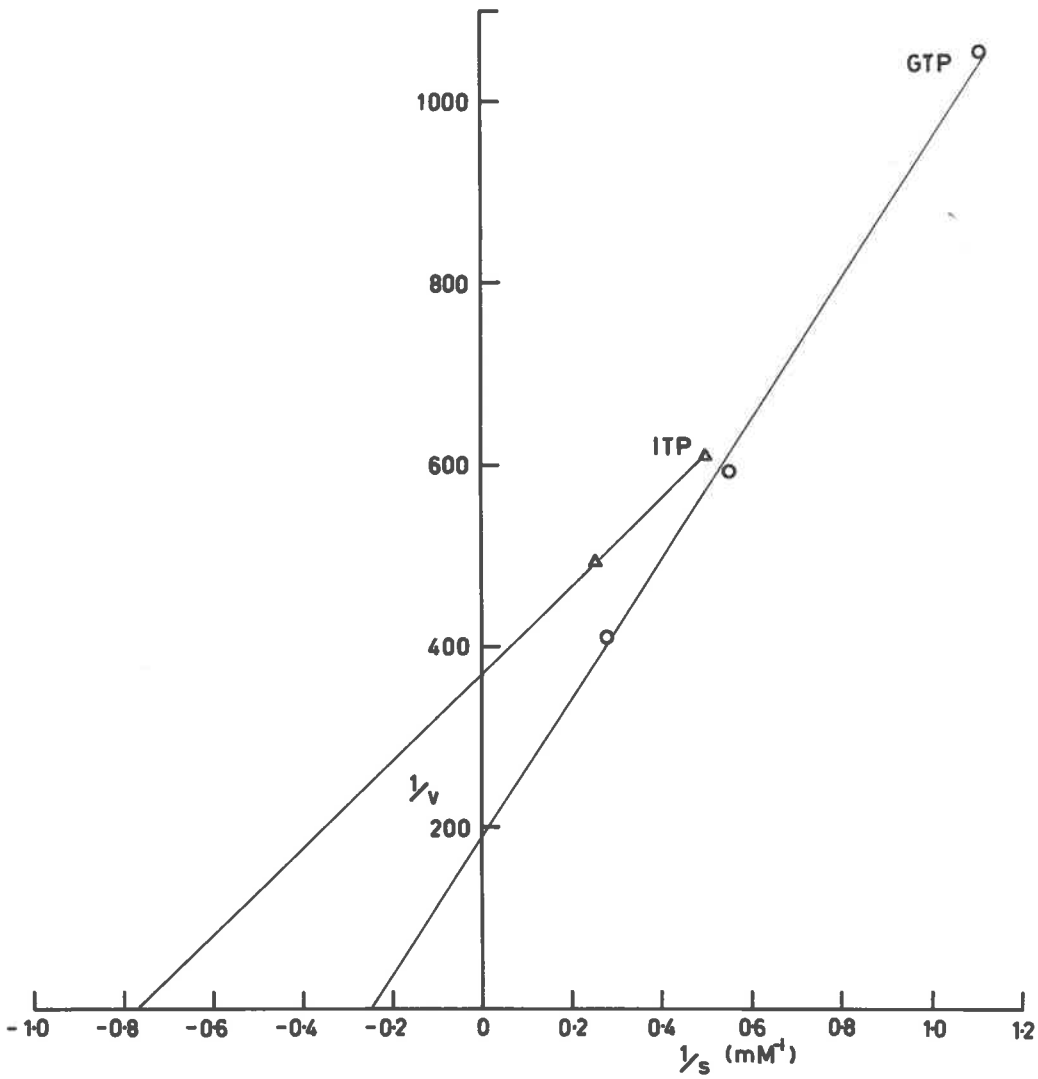
Using the coupled assay system (Methods (b)), the rate of guanylyl transfer to NMN was compared to the rate of formation of NAD and nicotinamide hypoxanthine dinucleotide from ATP and ITP respectively. The reaction was carried out at 25°, and was started by the addition of the ATP:NMN adenylyltransferase preparation where GTP or ITP was

present, transferase containing 990 γ of protein was used. With ATP only 99 γ of protein was necessary. All rates of dinucleotide formation were calculated as μmoles of NAD or analogue formed per minute per 990 γ of protein.

Table III-4 shows the results obtained. Under the same conditions, 0.164 μmoles of NAD were synthesized per minute per 990 γ of protein when ATP (1.64 mM) was used as the nucleotidyl donor. Figure III-5 compares the Lineweaver-Burk plots for rates of inosinyl and guanylyl transfer. It seems that ITP and GTP have similar kinetic parameters in the ATP:NMN adenylyl transferase reaction catalysed by the pig liver enzyme, although GTP reacts at a slightly higher rate than does ITP and has a higher K_m. The K_m obtained here for ITP (1.3 mM) by the coupled assay method is similar to that found using the discontinuous assay technique (1.7 mM).

Fig. III-5

A comparison of GTP and ITP as nucleotidyl donor to NMN.



A Comparison of GTP and ITP as Nucleotidyl Donor to NMN

Fig.III-5

Table III-4

A comparison of GTP and ITP as nucleotidyl donor to NMN.

Nucleoside triphosphate	Concentration (mM)	µMoles NAD analogue per minute per 990 Y protein
GTP	3.59	0.00244
K_m 4.0 mM		
	1.795	0.00169
V_{max} 0.00535 µmoles NAD analogue per minute per 990 Y protein	0.8975	0.00095
ITP	3.98	0.00203
K_m 1.3 mM		
	1.99	0.00163
V_{max} 0.00269 µmoles NAD analogue per minute per 990 Y protein		

(f) Inhibition of ATP:NMN adenylyl transfer by a 6-Mercaptopurine-riboside-5'-triphosphate preparation.

The 6-mercaptopurine-riboside-5'-triphosphate preparation (preparation A) was found to competitively inhibit ATP:NMN adenylyl-transferase. The non-coupled assay system (Methods (a)) was used for these inhibitory studies, the reaction being initiated by adding transferase to the reaction mixture containing substrates and inhibitor. After 5 minutes at 37°, the reaction was stopped and the amount of NAD

formed was estimated in the usual manner. The rates obtained in the presence and absence of preparation A and 6-mercaptopurine riboside-5'-monophosphate are indicated in Table III-5.

Table III-5

Inhibition of ATP:NMN adenylyltransferase by 6-mercaptopurine derivatives.

ATP concentration (mM)	Mercaptopurine riboside monophosphate concentration (mM)	Estimated * mercaptopurine riboside triphosphate concentration (mM)	µmoles NAD per minute
1.25	0	0	0.0260
1.25	0	0	0.0262
1.25	0.244	0	0.0264
1.25	0	0.124	0.0238
1.25	0	0.313	0.0196
2.5	0	0	0.0308
2.5	0.244	0	0.0310
2.5	0	0.124	0.0294
2.5	0	0.313	0.0256

* The estimated 6-mercaptopurine riboside-5'-triphosphate concentration in preparation A is based on the electrophoretic results and on the yields obtained by Roy et al. (1961). The total amount of 6-mercaptopurine nucleotides present (including the triphosphate) is twice that given above.

The inhibitory material (preparation A) contained 6-mercaptopurine riboside -5'-monophosphate, -5'-diphosphate and -5'-triphosphate (see Methods above), and was free of pyridine, dicyclohexylcarbodiimide and inorganic phosphates (including polyphosphates). As shown in Table III-5, 6-mercaptopurine riboside-5'-monophosphate is not inhibitory and it would seem unlikely that the corresponding diphosphate is the inhibitory factor. Assuming the triphosphate to be the cause of competitive inhibition, a Lineweaver-Burk plot of the rates of adenylyl transfer (figure III-6) indicated a K_i of 3.0 to 3.7×10^{-4}M for this compound. The K_m for ATP under the same conditions is 4×10^{-4}M (Table III-2). If, as seems likely (see Discussion), the inhibition is due to this nucleoside triphosphate analogue, then it has an extremely high affinity for ATP:NMN adenylyltransferase. These results have been confirmed subsequently with purified 6-mercaptopurine riboside-5'-triphosphate (Atkinson, Jackson, Morton and Murray, 1962).

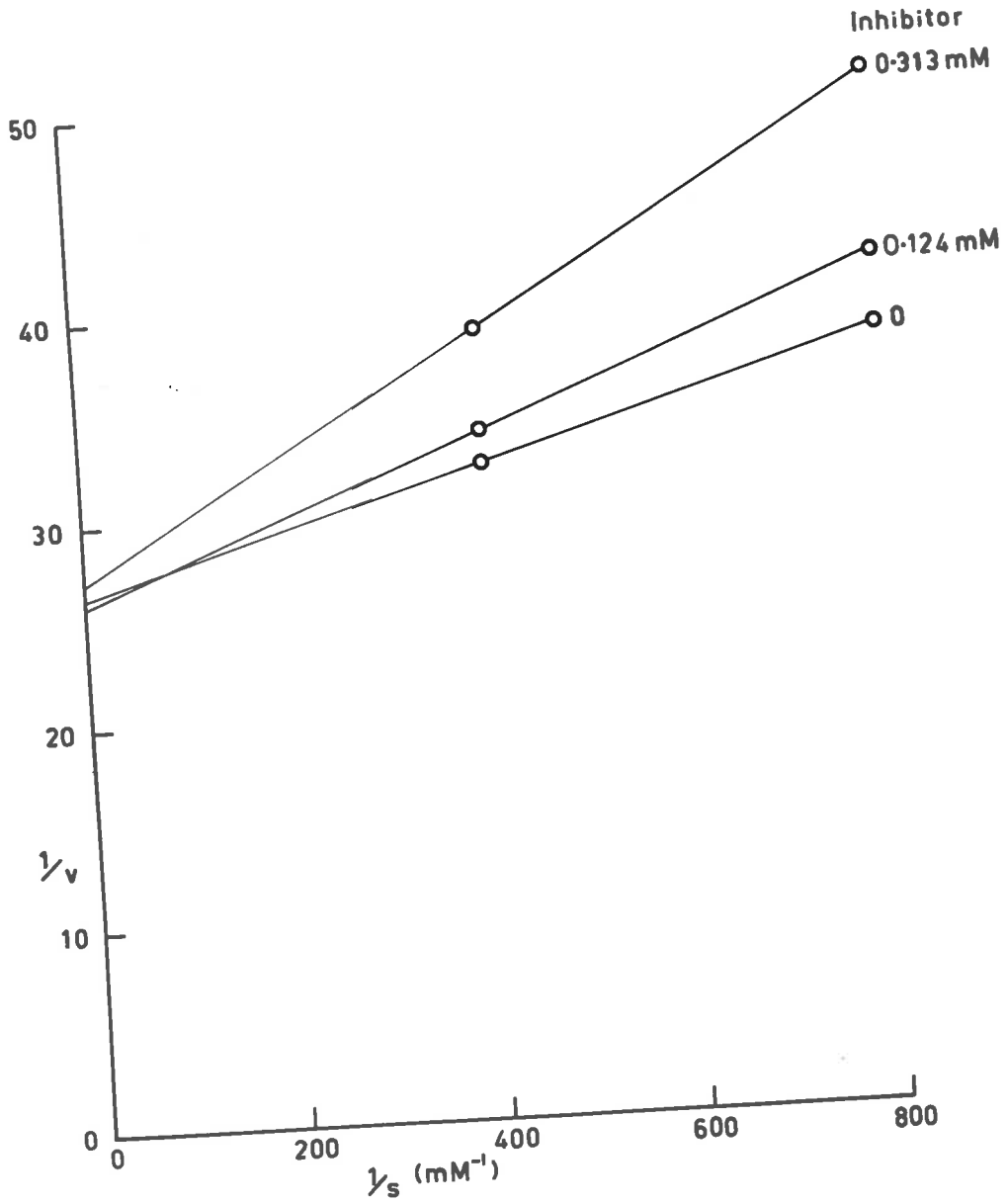
D. Discussion

A variety of ATP analogues have been tested for their ability to replace ATP in the reaction catalysed by pig liver ATP:NMN adenylyltransferase. In general the enzyme was found to be relatively specific, slight changes in the structure of the nucleoside triphosphate from that of ATP having a marked effect on the rate of nucleotidyl transfer to NMN. The findings are summarized in Table III-6.

Analysis by electrophoresis indicates that deoxy ATP is the best substitute for ATP as far as the overall rate of dinucleotide

Fig. III-6

Inhibition of ATP:NMN adenylyltransferase by 6-mercaptopurine riboside-5'-triphosphate



Inhibition of ATP:NMN Adenylyltransferase by
6-Mercaptopurine riboside-5′-triphosphate

Fig. III-6

Table III-6

The ability of ATP analogues to participate in the ATP:NMN adenylyltransferase reaction with NMN.

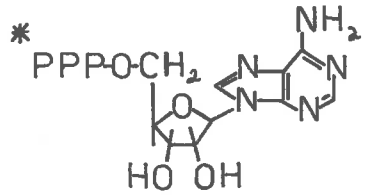
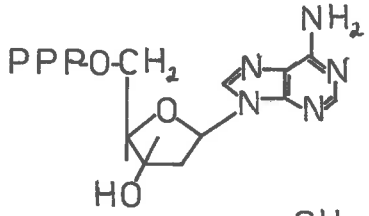
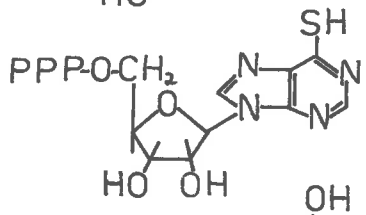
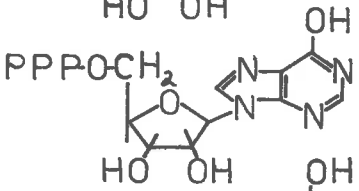
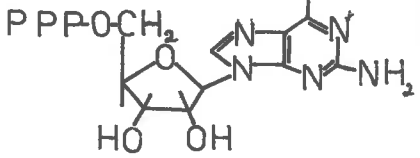
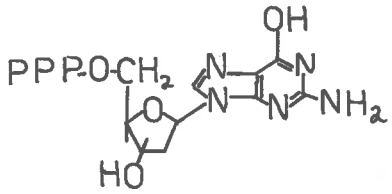
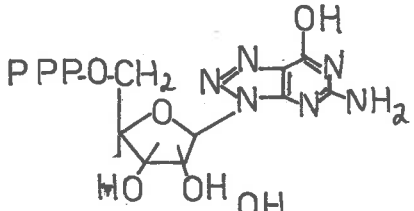
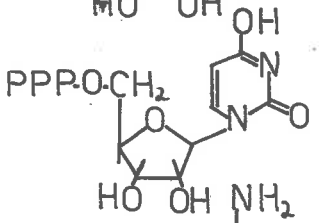
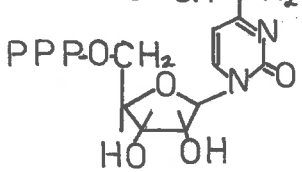
Structure	Nucleoside triphosphate	K_m (mM)	V_{max} (relative)	K_i (mM)	Remarks
	ATP	0.4	100	-	-
	deoxy ATP	-	-	-	Appears to be a good substitute for ATP
	6-mercapto-purine riboside-5'-triphosphate	-	-	0.35	Reacts slowly
	ITP	1.7	0.75	2.1	-
	GTP	4.0	1.5	-	-

Table III-6 contd.

Structure	Nucleoside triphosphate	K_m (mM)	V_{max} (relative)	K_i (mM)	Remarks
	deoxy GTP	-	-	-	Reacts very slowly
	8 aza GTP	-	-	-	Reacts very slowly
	UTP	-	-	-	No reaction
	CTP	-	-	-	No reaction

* Where PPP represents the triphosphate moiety.

synthesis is concerned. Alteration of the substituent at the 6 position of the purine ring of ATP appears to have a greater depressing effect on the rate of nucleotidyl transfer than does the removal of an hydroxyl group from the 2' position of the adenosine moiety. However, 6-mercaptopurine riboside-5' triphosphate and ITP each have a relatively low K_i (or K_m) value, perhaps reflecting a high affinity for pig liver ATP:NMN adenylyltransferase. Further alteration of the molecule by the introduction of an amino group at the 2 position of the purine ring (as in GTP) has a small but definite effect on the transfer reaction, GTP having a higher K_m and V_{max} than ITP. The ommission of an hydroxyl group in the ribose moiety of GTP (deoxy GTP), or the introduction of a nitrogen atom in place of carbon 8 (8 aza GTP), lowers the rate of reaction still further. Thus interaction with ATP:NMN adenylyltransferase is dependent on at least four points around the nucleoside triphosphate molecule. Substitution of a pyrimidine ring for the purine ring (as in UTP or CTP) results in complete loss of activity. In keeping with the conclusion that both the 6-amino group of the purine moiety and the 3-carboxamide side chain of the pyridine ring play an important part in the binding of substrate to the transferase, is the observation that the enzyme does not catalyse detectable pyrophosphorolysis of 3-pyridinealdehyde hypoxanthine dinucleotide or 3-acetylpyridine hypoxanthine dinucleotide. Here two changes have been made in the substrate molecule. This is in contrast to the finding that deoxy GTP, where three changes have been made in the ATP molecule, participated in the transfer reaction,

even though very slowly.

Deoxy ATP can also serve as a substrate for ATP:NMN adenylyltransferase from yeast (Klenow and Andersen, 1957), where it reacts at about 20 to 25% of the rate found for ATP at a concentration of 1mM. It is probable that this analogue reacts with the pig liver enzyme at about the same relative rate. Klenow and Andersen (1957) isolated the dinucleotide formed from deoxy ATP and NMN. Partial characterization of the product showed it to have the properties expected of deoxy NAD (P^1-nicotinamide riboside-5' P^2- deoxyadenosine-5' pyrophosphate).

Deoxyadenosine derivatives have been shown to be capable of replacing the corresponding adenosine derivatives in many other enzymic reactions. Thus deoxy ATP, one of the natural substrates for DNA polymerase (Lehman et al., 1958), can replace ATP in the myokinase reaction (Klenow and Andersen, 1957), and in the reactions catalysed by yeast hexokinase, phosphofructokinase, phosphoglycerate-kinase and pyruvic kinase (Siebert and Beyer, 1961). In each case the deoxy analogue reacted at a rate greater than 30% of that of ATP. Siebert and Beyer (1961) also found that deoxy ADP can be used as a phosphate acceptor in the glycolysis of rat liver extracts. Deoxy NAD, synthesized chemically from NMN and deoxy AMP, can be reduced by many NAD-dependent dehydrogenases (Honjo et al., 1962; Fawcett and Kaplan, 1962). Those capable of reducing the analogue include yeast and horse liver alcohol dehydrogenase, lactic dehydrogenase, malic dehydrogenase, glutamic dehydrogenase and triosephosphate dehydrogenase.

The rate of reduction of deoxy NAD varies from 9% to about 90% that of NAD with these enzymes. Klenow and Andersen (1957), who made the deoxydinucleotide enzymically, also found it to be reduced by yeast alcohol dehydrogenase and glutamic dehydrogenase.

In view of the relatively high rates with which deoxyribonucleotide analogues can participate in enzymic reactions normally thought to involve ribonucleotides, it is not surprising that many pyrophosphorylases, including ATP:NMN adenylyltransferase, can utilize deoxyribonucleoside triphosphates in the synthesis of dinucleotides. The rate of deoxycytidylyl transfer to choline-phosphate, catalysed by CTP: choline phosphate cytidylyltransferase, is approximately the same as that for cytidylyl transfer (Kennedy et al., 1959). The reaction is readily reversible; the K_m for CDP choline was found to be 2×10^{-4}M, and for deoxy CDP choline, 1×10^{-3}M. CTP:ethanolamine phosphate cytidylyltransferase preparations can use deoxy CTP at about 10% of the rate with CTP (Kennedy et al.,1954). Neufeld (1962) has found that UTP: α-D-glucose-1-phosphate uridylyltransferase from yeast will catalyse deoxyuridylyl transfer to α-D-glucose-1-phosphate. Two of the known pyrophosphorylases have a deoxyribonucleoside triphosphate as their natural substrate. They are TTP:α-D-glucose-1-phosphate thymidylyltransferase (Kornfeld and Glaser, 1961) and TTP: α-D-galactose-1-phosphate thymidylyltransferase (Pazur, Kleppe and Cepure, 1962).

ITP has been investigated in a number of enzyme systems for its ability to replace ATP. Kleinzeller (1942) showed that activity with this deaminated nucleotide is low, for many enzymes, compared to ATP, with the exception of myosin ATP'ase. Hexokinase is quite active

in the presence of ITP, where it has a K_m of 3.7×10^{-3}M (ATP K_m, 9.5×10^{-5}M) and a V_{max} approximately 30% that of ATP (Martinez, 1961). With muscle phosphoglycerate kinase, ITP has up to 40% the activity of ATP (Rao and Oesper, 1961). ATP: creatine transphosphorylase can also use ITP in place of ATP; with this enzyme it has a K_m of 130 mM (ATP K_m,0.4mM) and a V_{max} approximately the same as that for ATP (Nihei et al., 1961). In all these cases either the K_m is very high relative to that for ATP or the maximal velocity obtained in the presence of ITP approaches that for ATP. With ATP:NMN adenylyltransferase however, the K_m for ITP is relatively low and the V_{max} is less than 1% that obtained for ATP. This combination of circumstances makes ITP an effective inhibitor of nucleotidyl transfer to NMN.

A nucleoside triphosphate which should have similar properties to ITP is 6-mercaptopurine riboside-5'-triphosphate. This analogue has in fact also been shown to be capable of replacing ATP in the ATP:NMN adenylyltransferase reaction. The reaction was observed to proceed at a slower rate than with ATP, and the preliminary work described above indicated that this analogue competitively inhibited adenylyl transfer with a K_i of approximately the same value as the K_m for ATP, making it an extremely powerful inhibitor. By analogy with ITP it should have a K_m of about the same value as that for ATP.

More recently, 6-mercaptopurine-riboside-5'-triphosphate has been purified, and, in support of earlier work, found to be a very effective competitive inhibitor of adenylyl transfer (Atkinson et al. 1962). Using a coupled assay system, where the K_m for ATP was found

to be 7.4×10^{-5}M, the K_i for 6-mercaptopurine riboside 5'-triphosphate was 5×10^{-5}M. At a concentration of 0.4 mM, the thio analogue of ATP reacted at about 7% of the rate found with ATP in effecting nucleotidyl transfer to NMN.

6-Mercaptopurine derivatives have been implicated as potent inhibitors in a number of enzymic systems. Thus 6-mercaptopurine was found to be a substrate for xanthine oxidase, with a K_m of 17.5×10^{-6}M (xanthine: K_m, 5.4×10^{-6}M) and giving a maximal velocity approximately 1% of that obtained with xanthine. It competitively inhibited xanthine oxidation, with a K_i of 18×10^{-6}M (Silberman and Wyngaarden, 1961). 6-Mercaptopurine riboside 5'-phosphate inhibits conversion of IMP into AMP (Salser and Balis, 1959). The L-succino form of the 6-thio analogue of adenylsuccinic acid inhibits the cleavage of adenylsuccinic acid by partially purified adenylosuccinase, and is itself slowly and irreversibly cleaved (Hampton, 1962). That 6-mercaptopurine can be substituted for hypoxanthine or adenine moieties in enzymic reactions, is not without precedent. It has been shown that 6-mercaptopurine can replace hypoxanthine in the reaction catalysed by inosinic acid pyrophosphorylase from beef liver (Lukens and Herrington, 1957) and from E. coli (Carter, 1959). In addition, nicotinamide 6-mercaptopurine dinucleotide can be reduced by yeast alcohol dehydrogenase and dihydrolipoamide dehydrogenase, and 6-mercaptopurine riboside 5'-triphosphate can phosphorylate glucose in the presence of hexokinase (Atkinson et al., 1962). 6-Mercaptopurine riboside 5'-diphosphate is a powerful inhibitor of polynucleotide phosphorylase (Carbon, 1962).

The relatively specific character of the reaction catalysed by pig liver ATP:NMN adenylyltransferase is exemplified by the inability of UTP or CTP to replace ATP. This is in contrast to the dehydrogenases, where it is known that nicotinamide uracil dinucleotide can be reduced by yeast and horse liver alcohol dehydrogenase, beef liver glutamic dehydrogenase, rabbit muscle and beef heart lactic dehydrogenase and malic dehydrogenase from pig heart (Fawcett and Kaplan, 1962).

IV

The Effect of $MgCl_2$ Concentration of the Rate of ATP:NMN Adenylyl transfer

The effect of varying magnesium ion concentration on the rate of adenylyl transfer to NMN is described in this section. The results are interpreted in terms of the relative concentrations of the species ATP (uncomplexed) and MgATP complex.

A. Materials

$MgCl_2$ This was purchased from British Drug Houses Ltd. (A.R. grade).

ATP. ATP (best grade from Nutritional Biochemicals Corporation) was dissolved in water and titrated to pH 7.5 with sodium hydroxide before dilution to make two stock solutions 3.12 and 31.4 mM. The concentrations were checked by reading the optical density at 259 mμ.

NMN. A stock solution of 45.2 mM was used.

Glycylglycine. A solution of glycylglycine (British Drug Houses Ltd; laboratory reagent grade) was titrated to pH 7.5 with sodium hydroxide before dilution to 500 mM. An insoluble crystalline impurity in the preparation (possibly diketopiperazine) was removed by filtration.

Ethanol-glycine-sodium hydroxide mixture. This solution was 0.835M-ethanol - 0.417M-glycine - 0.416M-sodium hydroxide. The glycine used was from British Drug Houses, Ltd. (laboratory reagent grade).

ATP:NMN adenylyltransferase. The preparation used, after dialysis against 0.01M-sodium phosphate, pH 7.5, was capable of synthesizing 0.52 μmoles of NAD per minute per mg. of protein. It was stored frozen at -15°. Just before use it was thawed and 0.25 ml. was diluted to 1.5 ml. with ice-cold double distilled water.

The components of each reaction mixture were measured out with automatic-zero micropipettes. The enzymic reaction was carried

out in thick-walled tubes 10 cm. long x 1 cm. diameter.

B. Methods

Each reaction mixture contained 100 mM-glycylglycine, pH 7.5, 2.26 mM-NMN, and ATP and $MgCl_2$ at the concentration indicated. The reaction was initiated by the addition of 0.05 ml. of the ATP:NMN adenylyltransferase preparation, containing 54 γ of protein. The reaction was carried out at 37°, the total volume of the reaction mixture in each case being 1.0 ml. After 10 minutes the reaction was stopped with 1.5 ml. of 0.5M-trichloroacetic acid solution, and the precipitated protein removed by centrifugation. Two ml. of the supernatant was added to a spectrophotometer cell with a light path of 4 cm. After the addition of 3.0 ml. of the ethanol-glycine-sodium hydroxide solution, bringing the pH to about 9.5, the optical density of the resultant mixture was measured at 340 mμ before and after the addition of 75 γ of yeast alcohol dehydrogenase in 0.025 ml. (Boehringer). The spectrophotometer used was a manual CF4 Optica, fitted with a deuterium lamp. The difference in optical density was used to calculate the amount of NAD formed during the reaction, assuming a millimolar extinction coefficient of 6.22 at 340 mμ for the reduced coenzyme (Schrecker and Kornberg, 1948).

C. Results

Two series of experiments were carried out to observe the effect of varying $MgCl_2$ (and ATP) concentration on the rate of NAD synthesis by ATP:NMN adenylyltransferase. The results are given

Table IV-1

The effect of varying magnesium ion and ATP concentration on the rate of adenylyl transfer to NMN.

ATP (mM)	0.156	0.312	0.78	1.57	3.14	7.85	3.14	3.14	3.14	3.14	3.14	3.14
$MgCl_2$(mM)	20	20	20	20	20	20	0.1	0.2	0.5	1.0	2.0	5.0
µmoles NAD formed per minute	0.0088	0.0114	0.0144	0.0159	0.0167	0.0179	0.0051	0.0082	0.0125	0.0152	0.0152	0.0144

Table IV-2

The effect of varying magnesium ion and ATP concentration on the rate of adenylyl transfer to NMN.

ATP 1.57 mM								
$MgCl_2$(mM)	0.1	0.2	0.5	1.0	2.0	5.0	10.0	20.0
µmoles NAD formed per minute	0.0068	0.0095	0.0133	0.0138	0.0124	0.0138	0.0155	0.0160
ATP 3.14 mM								
$MgCl_2$(mM)	0.1	0.2	0.5	1.0	2.0	5.0	10.0	20.0
µmoles NAD formed per minute	0.0053	0.0079	0.0123	0.0145	0.0149	0.0142	0.0156	0.0170
ATP 7.85mM								
$MgCl_2$(mM)	0.1	0.2	0.5	1.0	2.0	5.0	10.0	20.0
µmoles NAD formed per minute	0.0032	0.0050	0.0090	0.0130	0.0154	0.0164	0.0163	0.0175

in Tables IV-1 and IV-2, and shown graphically in figures IV-1 and IV-2.

Under the conditions used here, the effect of varying $MgCl_2$ concentration does not follow the usual Michaelis-Menten rectangular hyperpobolic relationship between velocity and concentration. Instead there is a discontinuity in the region where the $MgCl_2$ and ATP concentrations are approximately equal. Below this region (i.e. at $MgCl_2$ concentrations less than about 1 mM), raising the ATP concentration at any particular level of $MgCl_2$ results in decreased NAD synthesis. Above this region (i.e. at $MgCl_2$ concentrations greater than 10 mM) raising the ATP concentration gives increased NAD synthesis. In contrast, the rates of adenylyl transfer obtained by varying ATP concentration at a fixed and relatively high $MgCl_2$ concentration (20 mM) conform to the usual Michaelis-Menten pattern.

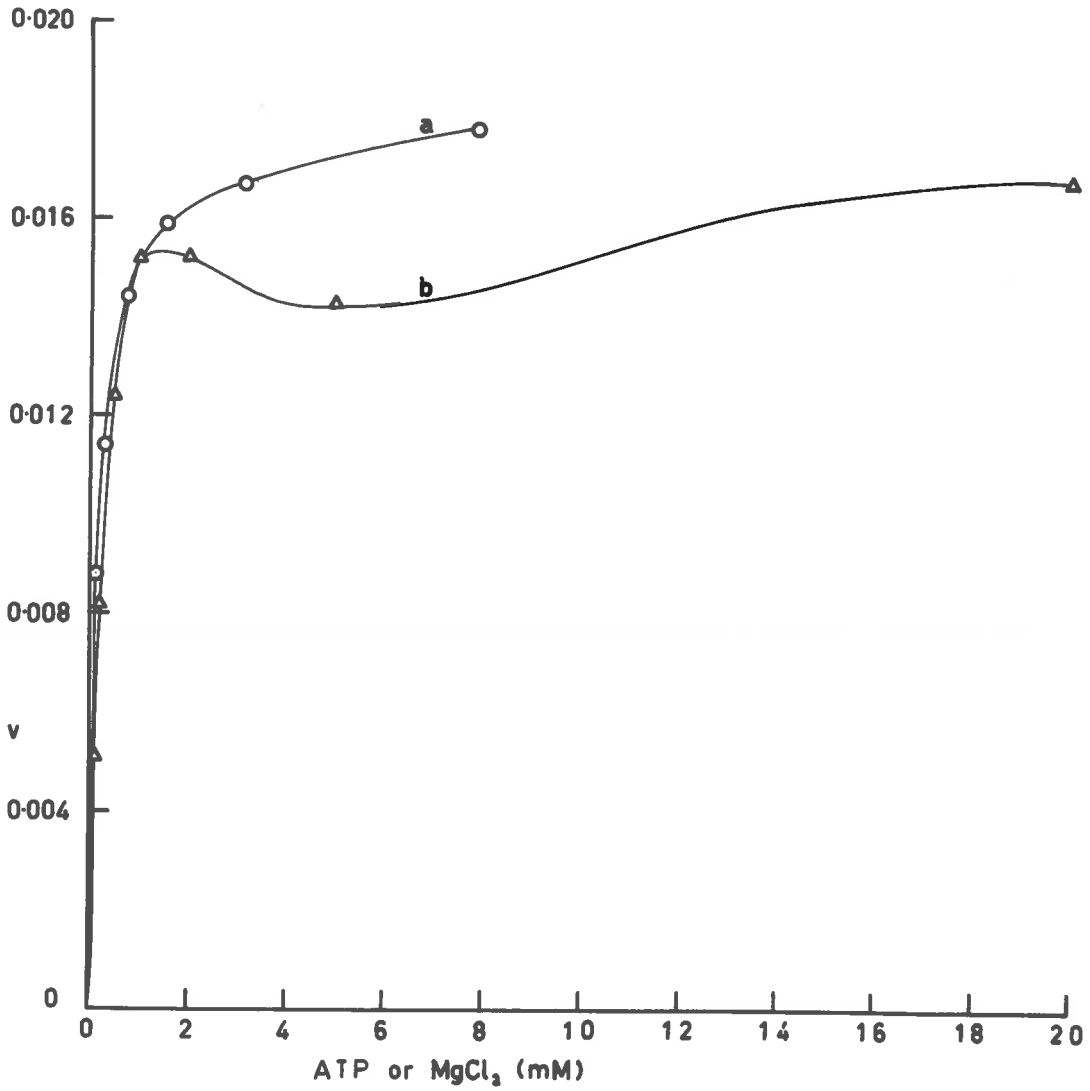
In seeking an explanation for these phenomena it would be of interest to know the relative concentration of ATP and MgATP complex in each of the above reaction mixtures. To do this an equation was derived, the solution of which permits the calculation of the concentration of each of these species, as well as that for Mg^{2+} and Mg glycylglycine complex. The essential steps in the derivation of this equation follow. Hydrogen ion ~~equation~~ concentration was not taken into account. The stability constant for MgATP was assumed to be 24.5 mM^{-1} (Nanninga, 1961) and that for Mg glycylglycine 0.0115 mM^{-1} (Chem. Soc., London, Special Publication number 6. 'Stability Constants Part I - Organic Ligands', 1957).

Fig. IV-1

Effect of $MgCl_2$ or ATP concentration on the transfer reaction.

(a) ATP concentration varied

(b) $MgCl_2$ concentration varied



Effect of $MgCl_2$ or ATP Concentration on the Transfer Reaction

Fig. IV - 1

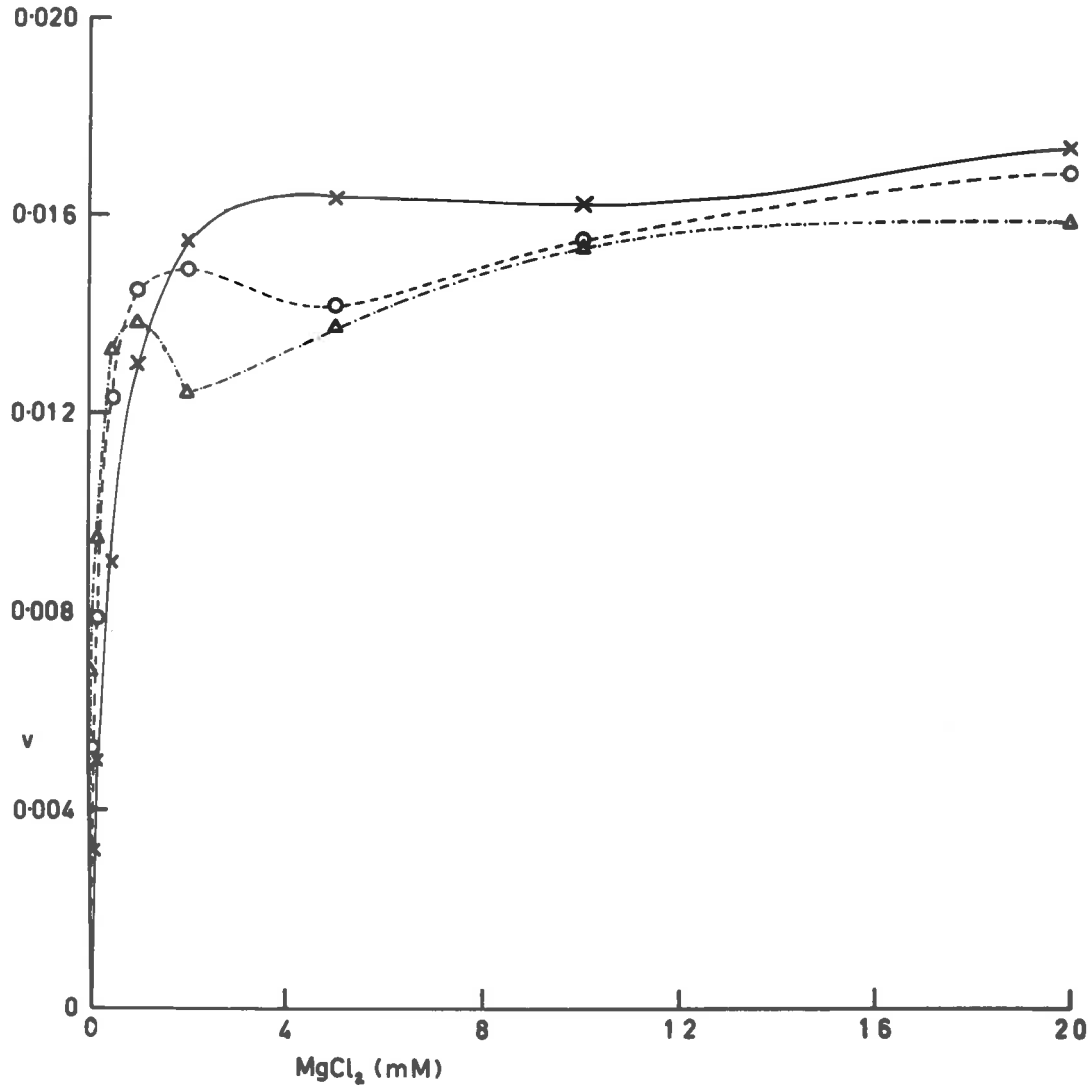
Fig. IV-2

Effect of $MgCl_2$ concentration on the transferase reaction

——————— 7.85 mM ATP

- - - - - - 3.14 mM ATP

-.-.-.-.-. 1.56 mM ATP



Effect of $MgCl_2$ Concentration
on the Transferase Reaction

Fig. IV-2

The symbols used are explained below.

Initially (mM)	Species	At Equilibrium ($_{m}M$)
a	Mg^{2+}	a-x-y
b	ATP	b-x
100	glycylglycine	100-y
0	Mg ATP	x
0	Mg glycylglycine	y

$$(1) \quad 24.5 = \frac{x}{(a-x-y)\ (b-x)}$$

$$(2) \quad 0.0115 = \frac{y}{(a-x-y)\ (100-y)}$$

from (1), $y = a-x-\frac{x}{(24.5)(b-x)}$

substituting in (2),

$$0.0115 = a-x-\frac{x}{24.5\ (b-x)}$$

$$(a-x-a+x+\frac{x}{24.5(b-x)})(100-a+x+\frac{x}{24.5(b-x)})$$

or (3)

$$x^3(599.72) - x^2\left[52.66 + (599.72)a + (1,199.72)b\right] + x\,b\left[52.675 + (1,199.72)a + 600\,b\right] - 600\,a\,b^2 = 0$$

Equation (3) was used to calculate the concentration of ATP and MgATP in the reaction mixtures indicated in Tables IV-1 and IV-2. The results are given in Tables IV-3 and IV-4 respectively. For comparison Table IV-5 and IV-6 show the ATP and MgATP concentrations calculated for the conditions in Table IV-1 and IV-2 when the presence of glycylglycine and metal ion complexing agents other than ATP has been ignored.

Inspection of Tables IV-3 to IV-6 shows that the presence of the weak magnesium ion-complexing ligand, glycylglycine, does depress the concentration of MgATP to a small extent. The concentration of the complex, Mg glycylglycine, is not high (with respect to MgATP) until most of the ATP has been complexed with magnesium ion.

Table IV-3

The concentration of ATP and MgATP for the conditions described in Table IV-1. Effects due to the presence of glycylglycine have been accounted for.

Total ATP (mM)	0.156	0.312	0.78	1.57	3.14	7.85	3.14	3.14	3.14	3.14	3.14	3.14
Total $MgCl_2$	20	20	20	20	20	20	0.10	0.20	0.5	1.0	2.0	5.0
MgATP	0.156	0.310	0.777	1.563	3.125	7.80	0.097	0.194	0.484	0.961	1.87	3.01
ATP	-	0.002	0.003	0.007	0.015	0.05	3.043	2.946	2.656	2.179	1.27	0.13

Table IV-4

The concentration of ATP and Mg ATP for the conditions described in Table IV-2. Effects due to the presence of glycylglycine have been accounted for.

Total ATP (mM)	1.57	1.57	1.57	1.57	1.57	1.57	1.57	1.57
Total $MgCl_2$	0.10	0.20	0.50	1.0	2.0	5.0	10.0	20.0
MgATP	0.094	0.188	0.463	0.886	1.377	1.531	1.554	1.563
ATP	1.476	1.382	1.107	0.684	0.193	0.039	0.016	0.007
Total ATP	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14
Total $MgCl_2$	0.10	0.20	0.50	1.0	2.0	5.0	10.0	20.0
MgATP	0.097	0.194	0.484	0.961	1.87	3.01	3.10	3.125
ATP	3.043	2.946	2.656	2.179	1.27	0.130	0.04	0.015

Table IV-4 contd.

Total ATP	7.85	7.85	7.85	7.85	7.85	7.85	7.85	7.85
Total $MgCl_2$	0.10	0.20	0.50	1.0	2.0	5.0	10.0	20.0
MgATP	0.099	0.198	0.494	0.988	1.971	4.858	7.581	7.800
ATP	7.751	7.652	7.356	6.862	5.879	2.992	0.269	0.050

Table IV-5

The concentration of ATP and MgATP for the conditions described in Table IV-1.

The presence of glycylglycine has been ignored.

Total ATP(mM)	0.156	0.312	0.780	1.57	3.14	7.85	3.14	3.14	3.14	3.14	3.14	3.14
Total $MgCl_2$	20	20	20	20	20	20	0.10	0.20	0.50	1.0	2.0	5.0
MgATP	0.155	0.311	0.778	1.567	3.132	7.824	0.099	0.197	0.492	0.981	1.935	3.075
ATP	0.001	0.001	0.002	0.003	0.008	0.126	3.041	2.943	2.648	2.159	1.205	0.065
Mg^{2+}	19.845	19.689	19.222	18.433	16.868	12.176	0.001	0.003	0.008	0.019	0.065	1.925

Table IV-6

The concentration of ATP and MgATP for the conditions described in Table IV-2.

The presence of glycylglycine has been ignored.

Total ATP(mM)	1.57	1.57	1.57	1.57	1.57	1.57	1.57	1.57
Total $MgCl_2$	0.10	0.20	0.50	1.0	2.0	5.0	10.0	20.0
MgATP	0.097	0.187	0.482	0.939	1.460	1.552	1.562	1.567
ATP	1.473	1.383	1.088	0.631	0.110	0.018	0.008	0.003
Mg^{2+}	0.003	0.013	0.018	0.061	0.540	3.448	8.438	18.433
Total ATP	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14
Total $MgCl_2$	0.10	0.20	0.50	1.0	2.0	5.0	10.0	20.0
MgATP	0.099	0.197	0.492	0.981	1.935	3.075	3.121	3.132
ATP	3.041	2.943	2.648	2.159	1.205	0.065	0.019	0.008
Mg^{2+}	0.001	0.003	0.008	0.019	0.065	1.925	6.239	16.868

Table IV-6 contd.

Total ATP	7.85	7.85	7.85	7.85	7.85	7.85	7.85	7.85
Total $MgCl_2$	0.10	0.20	0.50	1.0	2.0	5.0	10.0	20.0
MgATP	0.099	0.199	0.497	0.994	1.986	4.931	7.712	7.824
ATP	7.751	7.651	7.353	6.856	5.864	2.919	0.138	0.026
Mg^{2+}	0.001	0.001	0.003	0.006	0.014	0.069	2.288	12.176

The following is an attempt to explain the experimental results (Table IV-2) in terms of the thesis that MgATP is the active form of the substrate, using the data accumulated in Table IV-4. For convenience the results are considered in two sections; first, at concentrations where total $MgCl_2$ < total ATP, and second, where total $MgCl_2$ > total ATP.

(a) Total $MgCl_2$ < total ATP

Under these conditions, a higher concentration of total ATP at any one total $MgCl_2$ concentration gives rise to lower rates. This suggests that if MgATP is the true substrate for the transferase, then uncomplexed ATP is acting as a competitive inhibitor. This is a likely situation.

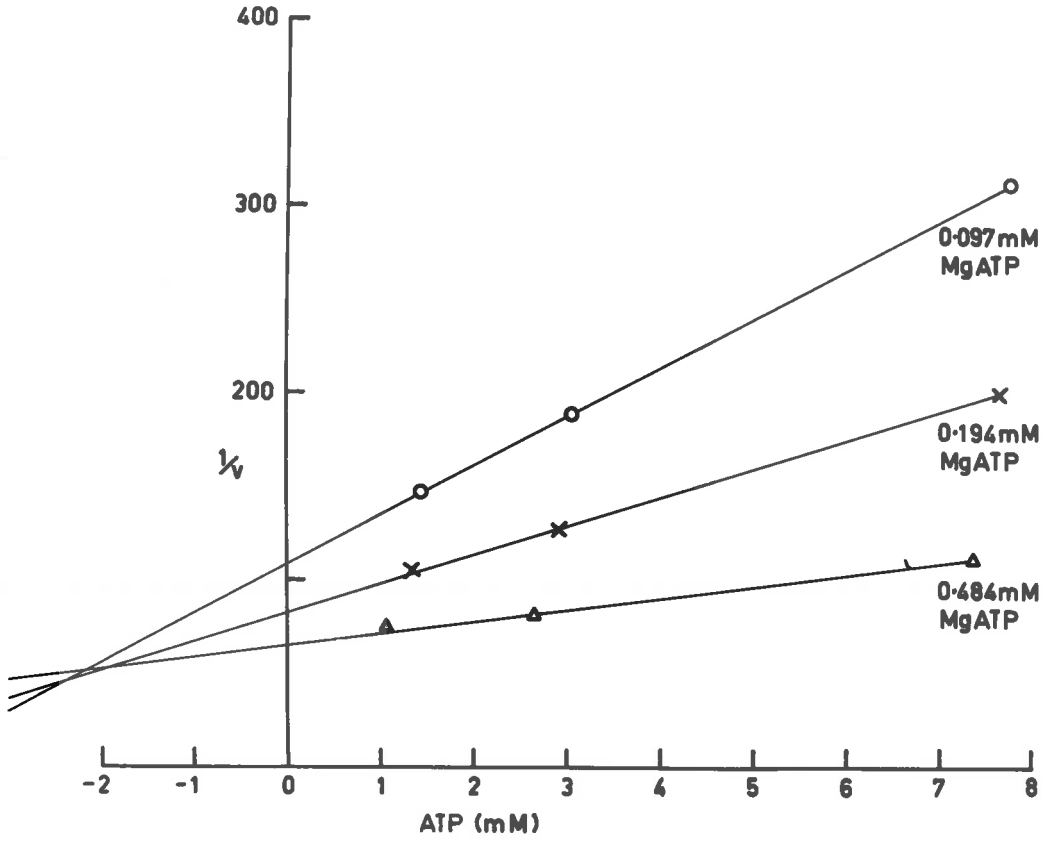
To test the hypothesis, the reciprocal of the velocity was plotted against uncomplexed ATP concentration at three levels of MgATP (0.099, 0.0194 and 0.484 mM) according to the method of Dixon (1953). As shown in figure IV-3, the result is consistent with this hypothesis. A K_i for ATP of 2.0mM, a K_m for MgATP of 0.12mM, and a V_{max} of 0.01925 μmoles NAD/min. was deduced from figure IV-3. The concentration of MgATP complex indicated in figure IV-3 are average values only, as they vary a little, depending on the total ATP concentration (see Table IV-4).

The velocity in the presence of a competitive inhibitor, i, and substrate, s, is given by

$$v = \frac{V_{max}}{1 + \frac{K_m}{s}\left(1 + \frac{i}{K_i}\right)} \qquad \ldots\ldots\ldots \quad (4)$$

Fig. IV-3

Inhibition of adenylyl transfer from MgATP by ATP.



Inhibition of Adenylyl Transfer from MgATP by ATP

Fig. IV - 3

Using equation (4) and the values for K_i, K_m and V_{max} indicated above, velocities were calculated for the set of conditions given in Table IV-4 and compared with the observed velocities (Table IV-2). The results are tabulated in Table IV-7 and shown graphically in figure IV-4. The solid lines in figure IV-4 represent calculated velocities and the observed velocities are indicated by points. There is fairly good agreement between calculated and observed velocities where total Mg < total ATP, but as expected, when total Mg ion concentration approaches total ATP concentration, the velocity falls away below that of the predicted value.

Again the hypothesis that ATP is a competitive inhibitor of MgATP under conditions where total Mg < total ATP, is in agreement with the kinetic behaviour of the system in these regions. This hypothesis also predicts that there will be a 'cross-over' of lines of different ATP concentration (see figure IV-5), so that in one region (Mg < ATP) increased ATP will give rise to lower rates and in the other region (Mg > ATP) increased ATP will give higher rates. This is also in agreement with the experimental results.

The results here would also be in accord with the presence of an inhibitor in the ATP preparation (e.g. inorganic pyrophosphate) or with the possibility that $HATP^{3-}$ is an inhibitor of the transferase If the pK'a of the dissociation

$$HATP^{3-} \rightleftharpoons ATP^{4-} + H^+$$

is assumed to have a value of 6.8 (Melchior, 1954; Smith and Alberty, 1956), then at pH 7.5 the ratio $ATP^{4-}/HATP^{3-}$ is 5.01. If $HATP^{3-}$ is

Fig IV-4

Effect of $MgCl_2$ concentration on ATP:NMN adenylyl transfer. The solid lines represent the relationship assuming inhibition by ATP not complexed with Mg ion. The points represent experimental values.

△ 7.85 mM ATP

× 3.14 mM ATP

○ 1.57 mM ATP

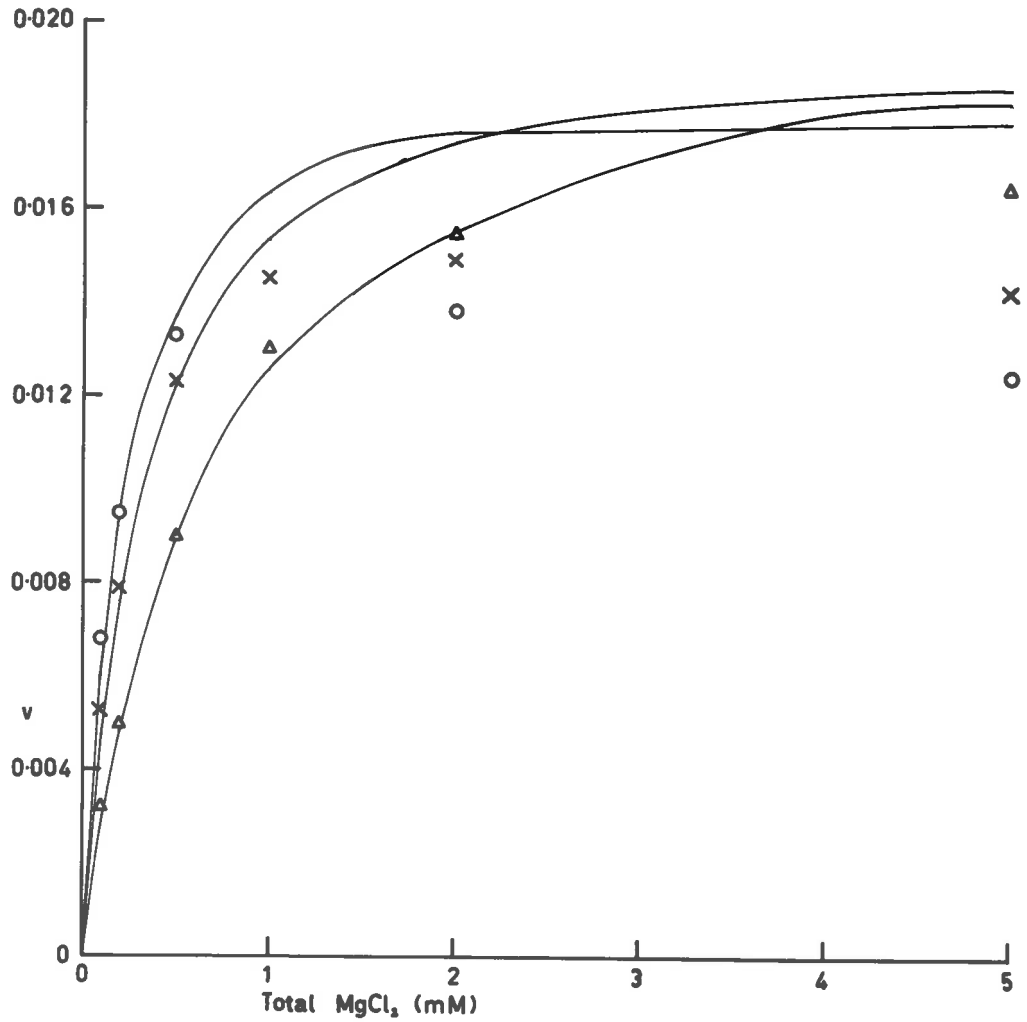


Fig. IV - 4

Fig. IV-5

Effect of $MgCl_2$ concentration on the rate of ATP:NMN adenylyl transfer, assuming inhibition by ATP not complexed with magnesium ion.

(a) 7.85 mM ATP

(b) 3.14 mM ATP

(c) 1.57 mM ATP

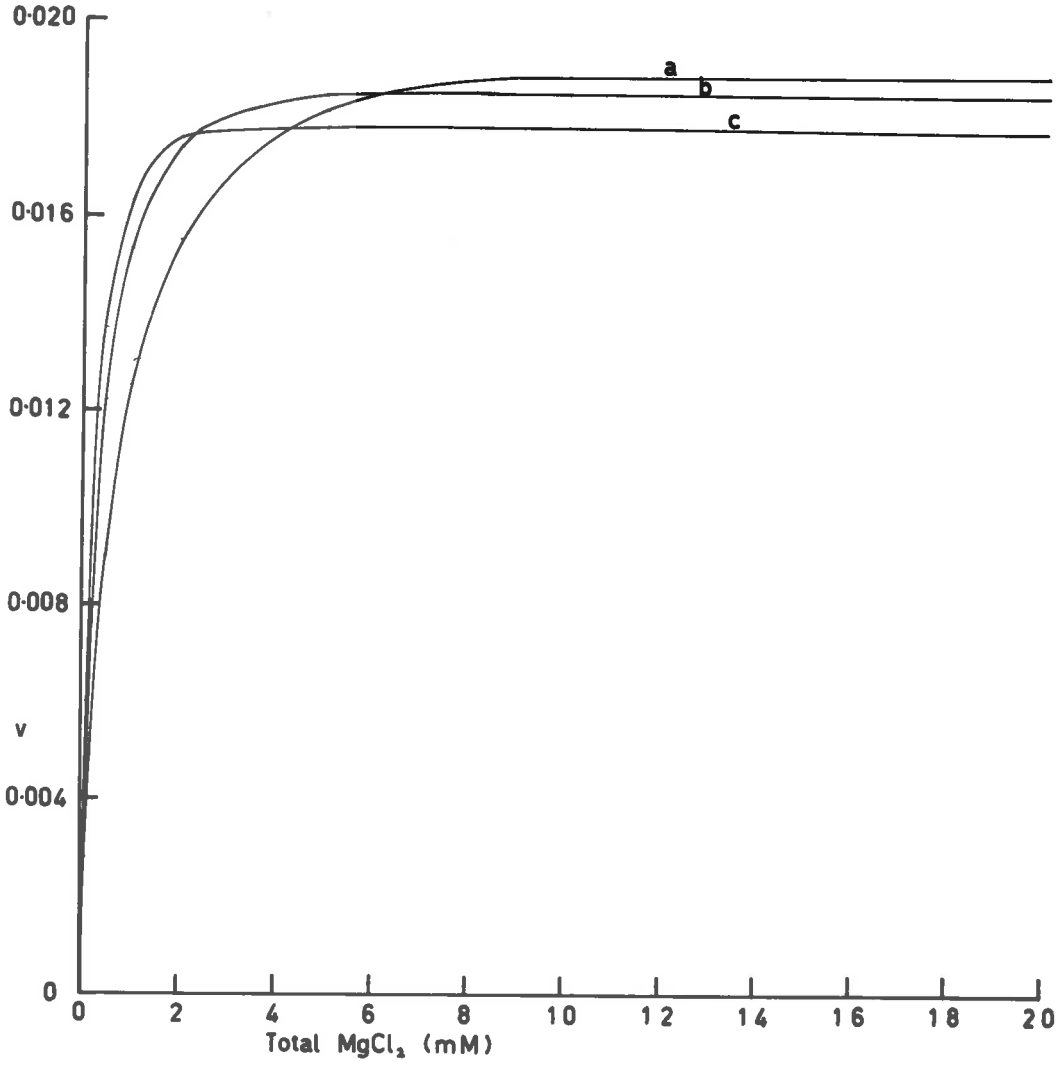

0·020
0·016
0·012
0·008
v
0·004
0
a
b
c
0
2
4
6
8
10
12
14
16
18
20
Total $MgCl_2$ (mM)

Fig. IV-5

Table IV-7

Correlation between the observed rate of adenylyl transfer and that predicted assuming competitive inhibition of transfer from MgATP by ATP.

ATP (mM)	MgATP (mM)	Predicted velocity (μmoles NAD/min.)	Observed velocity (μmoles NAD/min.)
Total ATP added 7.85 mM			
7.751	0.099	0.0028	0.0032
7.652	0.198	0.0049	0.0050
7.356	0.494	0.0090	0.0090
6.862	0.988	0.0125	0.00130
5.879	1.971	0.0155	0.0154
2.992	4.858	0.0181	0.0164
0.269	7.581	0.0189	0.0163
0.050	7.800	0.0190	0.0175
Total ATP added 3.14 mM			
3.043	0.097	0.0047	0.0053
2.946	0.194	0.0076	0.0079
2.656	0.484	0.0122	0.0123
2.179	0.961	0.0153	0.0145
1.270	1.870	0.0174	0.0149
0.130	3.010	0.01845	0.0142
0.040	3.100	0.0185	0.0156
0.015	3.125	0.0186	0.0170

Table IV-7 contd.

ATP (mM)	MgATP (mM)	Predicted velocity (μmoles NAD/min.)	Observed velocity (μmoles NAD/min.)
Total ATP added 1.57 mM			
1.476	0.094	0.0060	0.0068
1.382	0.188	0.00925	0.0095
1.107	0.463	0.0137	0.0133
0.684	0.886	0.0163	0.0138
0.193	1.377	0.0176	0.0124
0.039	1.531	0.0178	0.0138
0.016	1.554	0.0179	0.0155
0.007	1.563	0.0179	0.0160

the only inhibiting species present in the reaction mixtures, then it would be a competitive inhibitor with a K_i of 0.4 mM. These two cases cannot be ruled out with the present experimental results; however a study of the kinetics at several hydrogen ion concentrations would test the second case.

(b) Total $MgCl_2$ > total ATP

Table IV-1 and figure IV-1 show the velocities obtained when ATP concentration is varied and Mg^{2+} concentration maintained at a

high constant value (20 mM). They indicate a $K_{m_{MgATP}}$ of 0.171 mM and a V_{max} of 0.01755 μmoles NAD/min. These kinetic parameters are different from those noted above, and indicate lower velocities. However, this experiment was carried out under conditions where total Mg > total ATP. This may suggest that in passing from a region where Mg < ATP to one where Mg > ATP, as in figure IV-2, there would be a general lowering of rates, giving rise to a discontinuity similar to that in Figure IV-2.

The reason for the lowering of rates is obscure. It could be due to inhibition by Mg_2ATP or by Mg glycylglycine, or simply a change of mechanism whereby an enzyme-Mg complex binds MgATP. However if it was solely due to inhibition by Mg_2ATP or Mg glycyl-glycine, then one would expect the rates to progressively decrease as $MgCl_2$ concentration is increased. The reverse is the case (figure IV-2), the rates actually increasing as $MgCl_2$ is increased. This would seem to leave a change of mechanism as at least part of the explanation.

To sum up then, the results embodied in figure IV-2 can be explained by assuming that MgATP is the substrate for the transferase. Where total Mg < total ATP, ATP acts as a competitive inhibitor, causing rates to be lowered when ATP concentration is raised at any particular $MgCl_2$ concentration. When ATP has been removed from the system by an excess of $MgCl_2$ (i.e. when total Mg > total ATP), there is a 'crossover' of lines representing different ATP

concentrations (total) causing a higher rate when total ATP is increased at any one $MgCl_2$ concentration. In passing from the region where total Mg < total ATP to one where total Mg > total ATP, there is a change-over in mechanism, resulting in a general lowering of rates (higher K_m, lower V_{max}) and the discontinuity observed in figure IV-2. The nature of this mechanism change is not clear.

It is of interest here, that Dr. M. R. Atkinson, using a coupled assay system (i.e. coupling the transferase reaction with that of yeast alcohol dehydrogenase) at pH 8.0 for measuring initial rates, found that Michaelis-Menten kinetics were approximately obeyed when varying total $MgCl_2$ concentration (0.4 to 0.04 mM) at constant total ATP (3 mM) or when varying total ATP (0.4 to 0.04 mM) at constant $MgCl_2$ (3mM). This is consistent with the above conclusion, as at the low concentration of $MgCl_2$ used in the first experiment (0.4 to 0.04 mM) the concentration of ATP (competitive inhibitor) is almost constant. He also noted that higher rates were obtained in this experiment where total Mg < total ATP, compared with the second where total Mg > total ATP. In this coupled assay the total concentrations of ATP, Mg, or NMN needed to give half the rates found on extrapolation to infinite reactant concentrations were: 7.1×10^{-5}M -Mg, 7.1×10^{-5}M-ATP and 3.9×10^{-5}M-NMN. In each case the total concentration of each reactant which was not being varied was 3mM. The apparent equality of the K_m for ATP and the concentration of all magnesium species for half maximum rate is consistent with mechanisms in which MgATP is a substrate (Dixon and Webb, 1958, p.462)

D. Discussion

A number of enzymes using ATP as substrate and Mg^{2+} as the activating ion show a striking dependence on the ratio of Mg^{2+} to ATP. Usually maximum rates are obtained when the Mg^{2+}/ATP ratio is of the order of one to two, with the velocity falling away on either side of this maximum. Such behaviour is shown by fructokinase (Hers, 1952), hexokinase (Liebecq, 1954), gluconokinase (Leder, 1957), phosphohexokinase (Lardy and Parks, 1956), actomyosin ATP'ase (Perry and Grey, 1956) and ATP: creatine transphosphorylase (Kuby, Noda and Lardy, 1954). The last group of authors found a similar relationship between velocity and $MgSO_4$ concentration to that described above for ATP:NMN adenylyltransferase. They concluded that Mg-ATP is the reactive component, and that at a high concentration of $MgSO_4$, Mg_2-ATP is formed. This latter complex was assumed to be somewhat less reactive than the former.

A portion of this work on ATP:creatine transphosphorylase has been repeated by Noda, Nihei and Morales (1960), using magnesium acetate instead of $MgSO_4$. They give data which show sulphate ion to be a competitive inhibitor with respect to ATP. This is thought to be responsible for the discontinuous increase in the rate of transphosphorylation as the $MgSO_4$ concentration is increased. The use of magnesium acetate certainly did appear to smooth out the curve somewhat, but inspection of their result show that they have only considered a relatively narrow span of magnesium ion concentration. In the case

of ATP:NMN adenylyltransferase, at concentrations of $MgCl_2$ higher than that at the discontinuous region, the velocity increases once more with increasing $MgCl_2$ concentration. This is hard to reconcile with the possibility that chloride ions are inhibitory.

Inorganic pyrophosphatase shows a similar type of behaviour to the above enzymes. Here again pyrophosphate has a high tendency to form complexes with magnesium and other divalent ions (Wolhoff and Overbeek, 1959). Yeast inorganic pyrophosphatase, whether hydrolysing inorganic pyrophosphate in the presence of Mg ions or hydrolysing ATP in the presence of Zn ions, shows a maximum velocity where the ratio of divalent ion to polyphosphate is about one to two (Schlesinger and Coon, 1960; Kunitz, 1962). Inorganic pyrophosphatase, purified from rat brain, shows another similarity with ATP:NMN adenylyltransferase (Robbins, Stulberg and Boyer, 1955). It was found that at all concentrations, addition of pyrophosphate in excess of Mg^{2+} concentration results in decreased velocity, indicating competitive inhibition by uncomplexed pyrophosphate. The results of rate studies could be explained by assuming a K_i of 5×10^{-5}M for uncomplexed pyrophosphate, and a K_m of 1.72×10^{-4}M for the Mg-pyrophosphate complex.

Many of the pyrophosphorylases exhibit this dependence on the ratio of Mg^{2+} ion to polyphosphate (nucleoside triphosphate or pyrophosphate). Among those involved are ATP:FMN adenylyltransferase (De Luca and Kaplan, 1958; Schrecker and Kornberg, 1950), CTP: cholinephosphate cytidylyltransferase (Borkenhagen and Kennedy, 1957), UTP: α-D-glucose-1-phosphate uridylyltransferase (Munch-Petersen, 1955; Turner and Turner, 1958), UTP:N-acetylglucosamine-1-phosphate

uridylyltransferase (Strominger and Smith, 1959) and TTP: α-D-glucose-1-phosphate thymidylyl transferase (Kornfeld and Glaser, 1961). In all these cases, inhibition is shown at a ratio of Mg^{2+}/polyphosphate greater than about one or two. So far as is known, none of them show the recovery of velocity that is shown by ATP:NMN adenylyltransferase at higher Mg^{2+} concentrations.

V

The Specificity of the Metal Ion Requirements for the ATP:NMN Adenylyltransferase Reaction.

The cations of magnesium, nickel, cobalt, zinc and manganese have been found to satisfy the metal ion requirements for the ATP:NMN adenylyltransferase reaction. The maximum rates obtained with these cations can be correlated with the heat of hydration of the divalent metal ion. Possible explanations for the role of the cation in this and related transfer reactions are discussed.

A. Materials

$BeCl_2$ was purchased from British Drug Houses Ltd. as a 20% (w/w) aqueous solution. It had a specific gravity of 1.134 g./ml. A stock solution of concentration 200mM was used for the experiments described below.

$MgCL_2$ (Analar;A.R.), $NiCl_2$ (Analar;A.R.), $CoCl_2$ (B.D.H.; A.R.) $MnCl_2$ (Analar; A.R.) and $ZnSO_4$ (Analar; A.R.) were used as aqueous solutions of concentration 2, 20 and 200 mM.

ATP (best grade from Boehringer or from Nutritional Biochemicals Corporation) was used from stock solutions, pH 7.6. The concentrations were checked by spectrophotometry at 259 mμ before use.

ITP was purchased from Sigma Chemical Co., The stock solution was 50 mM, pH 7.6

Glycylglycine was obtained from Sigma Chemical Co. and from British Drug Houses Ltd. The commercial source of this buffer will be indicated in the experiments described below. That obtained from Sigma did not have the insoluble impurities present in the other preparation. Stock solutions were 1M or 0.5M, titrated to pH 8.0, 7.6 or 7.5 with sodium hydroxide solution.

Tris(hydroxymethyl) amino methane from Nutritional Biochemicals Corporation was used as a stock solution titrated to pH 7.4 or 7.6 with an HCl solution.

Ethylene diamine tetraacetic acid (B.D.H.). After titration to pH 8.0 with sodium hydroxide, the stock solution was diluted to 200mM before use.

Yeast alcohol dehydrogenase was purchased from either Sigma or Boehringer.

NMN. A stock solution of 45.2 mM was used.

Other reagents used are described in Section IV.

B. Methods

The composition of each reaction mixture used will be indicated as each experiment is described. All reactions were carried out at 37°. The procedure adopted for stopping the enzymic reaction and estimating the amount of dinucleotide formed was the same as described in Section IV. Where nicotinamide hypoxanthine dinucleotide was estimated, a more concentrated solution of dehydrogenase was used. This dinucleotide is reduced more slowly than NAD in the presence of yeast alcohol dehydrogenase (Pullman, Colowick and Kaplan, 1952).

C. Results

(a) Preliminary Experiments

In the presence of 200mM glycylglycine (Sigma), pH 7.6, 4.52 mM-NMN and 4 mM-ATP (Boehringer), the metal ion specificity of ATP:NMN adenylyltransferase was tested using 20 mM-$MgCl_2$, $NiCl_2$, $CoCl_2$, $MnCl_2$ and $ZnSO_4$. The reaction was started by the addition of 0.02ml. of a preparation of the transferase containing 198 γ of protein. (specific activity 0.54 μmoles NAD synthesized per minute per mg. protein). After four minutes at 37°, the reaction was stopped and the NAD synthesized was estimated in the usual manner. Table V-1 shows the results of two experiments carried out in the same way. The rates of NAD synthesis indicated in this table are relative to one of the

two cases in which $MgCl_2$ was used as the metal ion component. In this case, 0.0321 μmoles of NAD were synthesized every minute. In the absence of divalent metal ion, no NAD was synthesized. Where $MnCl_2$ or $ZnSO_4$ was present, a slight precipitate was observed to form during the incubation period.

Table V-1

Relative activities of some divalent metal ions in the ATP:NMN adenylyltransferase reaction.

Metal ion (20mM)	Mg	Ni	Co	Mn	Zn
Relative activity (i)	1.00	3.95	2.54	0.33	0.73
(ii)	1.12	3.86	2.66	0.32	0.68
		3·45	2·38	0·29	0·61
	1·00	3·70	2·46	0·31	0·67

(b) Metal ion specificity of inosinyl transfer from ITP to NMN, catalysed by ATP:NMN adenylyltransferase

The reaction mixtures in this case consisted of 200 mM glycyl-glycine (Sigma), pH 7.6, 4.52 mM-NMN, 5mM-ITP and 20 mM-metal ion. The reaction was started, as before, by a solution containing ATP:NMN adenylyltransferase (198 γ Of protein; specific activity 0.54 μmoles NAD synthesized per minute per mg. protein). The reaction was carried out at 37° for 30 minutes, and the nicotinamide hypoxanthine dinucleotide so formed estimated as for NAD. Under these conditions in the presence of 20 mM-$MgCl_2$, 0.00112 μmoles of nicotinamide hypoxanthine dinucleotide were synthesized per minute. Table V-2 shows the relative

activities of the various divalent ions in aiding inosinyl transfer to NMN in the presence of the adenylyl transfering enzyme. The relative activities are not very different from those observed for adenylyl transfer listed in Table V-1 above.

Table V-2

Relative activities of some divalent metal ions in the ITP:NMN inosinyl transfer reaction.

Metal ion (20mM)	Mg	Ni	Co	Zn
Relative activity	1.00	3.20	1.91	0.50

As observed in (a) above, a precipitate appeared during the enzymic reaction in the case where Zn ions were present.

(c) The relative activities of $MgCl_2$ and $NiCl_2$ in activating the ATP:NMN adenylyltransferase reaction of a pig-liver homogenate.

A pig-liver homogenate was obtained by homogenising 100 g. (wet weight) of sliced pig liver with 400 ml. of 0.25M-sucrose-0.003M-$CaCl_2$ (precooled to 2°) in a Waring blendor. It was filtered through hospital gauze before use, to rid it of coarser material. To test the homogenate for its transferase activity in the presence of $MgCl_2$ or $NiCl_2$, the reaction mixtures used contained 200 mM-glycylglycine (Sigma), pH 7.6, 4.52mM-NMN, 4mM-ATP and metal ions as indicated. Synthesis of NAD was initiated by the addition of 0.2 ml.

of homogenate. Adenylyl transfer was allowed to proceed at 37° for five minutes, the reaction was stopped, and the synthesized NAD was estimated as before. The results are tabulated in Table V-3.

Table V-3

Relative activities of $MgCl_2$ and $NiCl_2$ in activating the transferase reaction of a pig liver homogenate.

	1	2	3	4
Metal ion	$MgCl_2$(20mM)	$MgCl_2$(20mM) + $NiCl_2$(20mM)	$NiCl_2$(20mM)	none
µmoles NAD per min.	0.0180	0.0206	0.0308	0.0113

Here again more NAD was formed in the presence of $NiCl_2$ than with $MgCl_2$, although the effect is less marked than with the enzyme partially purified from pig liver. As there is some synthesis of NAD when no divalent metal (other than Ca^{2+}) is added to the system, it is probable that there are some metal ions in the homogenate. This, together with the possibility that Ni ions are bound by the protein and other molecules present in the homogenate to a larger extent than are Mg ions, may account for the smaller magnitude of activation by $NiCl_2$. However, the presence of $CaCl_2$ in the homogenising medium introduces Ca ions into the reaction mixture to a final concentration of 0.48 mM. As noted in the discussion to follow, although Ca ions are not active in promoting NAD synthesis in the presence of the transferase, they have

not been tested for any possible inhibition of the system.

(d) Kinetics of the metal ion specificity of the transferase at pH 8.0 using glycylglycine buffer.

Experiments were carried out to compare the kinetic parameters for $MgCl_2$, $NiCl_2$, $CoCl_2$, $ZnSO_4$ and $MnCl_2$ at a constant high concentration of ATP with those obtained for ATP at a constant, high concentration of either $MgCl_2$, $NiCl_2$, $CoCl_2$, $ZnSO_4$ or $MnCl_2$.

As indicated in section IV above, variation of $MgCl_2$ concentration does not give rates in accord with the usual Michaelis-Menten pattern, and this may be so with the other metal ions which are active in the ATP:NMN adenylyltransferase system. However, in order to gain some idea of the relative apparent K_m and V_{max} for these various metal ions, the transferase was incubated with 0.2, 2 and 20 mM concentrations of either $MgCl_2$, $CoCl_2$, $NiCl_2$, $MnCl_2$ and $ZnSO_4$. Besides these materials the reaction mixture contained 2.26 mM-NMN, 3.02 mM-ATP and 200 mM-glycylglycine (B.D.H.), pH 8.0, in a total volume of 1.0 ml. Just before use, a stock solution of transferase containing 5.4 mg. protein per ml. was thawed and a small portion diluted six-fold with 0.01M-sodium phosphate, pH 7.6. The reaction was started by the addition of 0.05 ml. of this preparation, containing 45 γ of protein (specific activity 0.5 μmoles NAD synthesized per minute per mg. protein). To ensure that approximately the same amount of NAD was synthesized in each case, the reaction was allowed to proceed for three minutes in the case of $NiCl_2$, five minutes for $CoCl_2$, 10 minutes for $MgCl_2$ and $ZnSO_4$, and 16 minutes for $MnCl_2$.

The reaction was stopped and the synthesized NAD estimated in the usual manner.

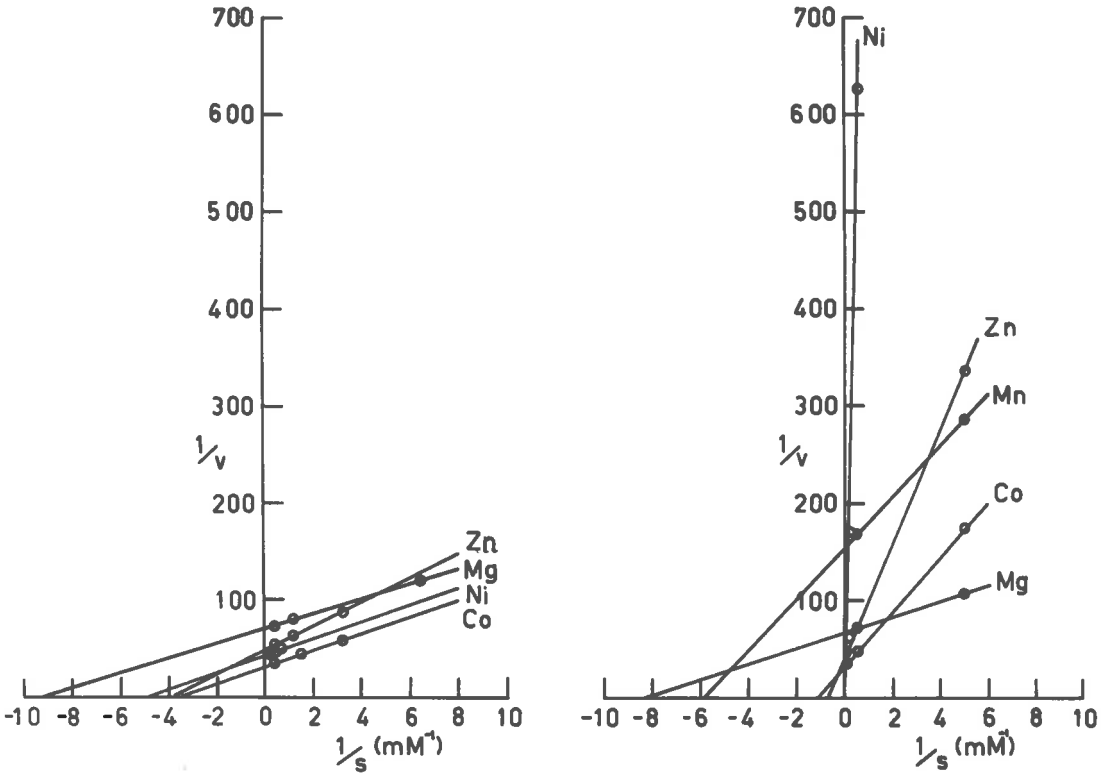
The rates so obtained fell approximately on a straight line for each metal, when plotting the data according to the method of Lineweaver and Burk, (1934). This enabled apparent K_m and V_{max} values to be assigned to each of the divalent metal ions used.

These kinetic parameters were then compared with those obtained by varying ATP concentration in the presence of constant amounts of either $MgCl_2$(4mM), $NiCl_2$(20mM), $CoCl_2$(10mM), $ZnSO_4$(10mM) or $MnCl_2$(4mM). Again, the reaction mixture also contained 2.26mM-NMN and 200mM-glycylglycine (BDH), pH8.0. The reaction was initiated by the addition of the same transferase preparation used above. Where $NiCl_2$ or $CoCl_2$ was used as cofactor, NAD synthesis was allowed to proceed for five minutes; for $ZnSO_4$ and $MgCl_2$, 10 minutes and for $MnCl_2$, 16 minutes elapsed before the reaction was stopped. Rates were calculated from the amount of NAD synthesized and plotted according to the method of Lineweaver and Burk (1934).

The results of the two sets of experiments are given in Table V-4. The apparent kinetic parameters indicated in this table were calculated from the double reciprocal plots shown in figure V-1. The velocities of the enzymic reaction obtained in the presence of $MnCl_2$ are unreliable, as in most cases a precipitate developed in the reaction mixtures on standing. This also occurs occasionally where $ZnSO_4$ is a component of the reaction mixtures. Because of this, most emphasis is given to the kinetics of the reaction in the presence of $MgCl_2$, $CoCl_2$ and $NiCl_2$.

Fig. V-1

Kinetics of the metal ion specificity of ATP:NMN adenylyl transfer.



ATP Varied
Metal Ion Constant

ATP Constant
Metal Ion Varied

Fig. V -1

Metal ion specificity of the ATP-NMN adenylyltransferase reaction

Table V-4

Kinetics of metal ion specificity at pH8.0 using glycylglycine buffer.

$MgCl_2$(mM)	20	2	0.2	4	4	4
ATP (mM)	3.02	3.02	3.02	0.157	0.628	3.02
v(μmoles NAD /min.)	0.0177	0.0143	0.0094	0.0083	0.0121	0.0135
$NiCl_2$ (mM)	20	2	0.2	20	20	20
ATP (mM)	3.02	3.02	3.02	1.51	3.02	7.55
v(μmoles NAD /min.)	0.0199	0.0016	0	0.0198	0.0212	0.0218
$CoCl_2$(mM)	20	2	0.2	10	10	10
ATP (mM)	3.02	3.02	3.02	0.314	0.628	3.02
v(μmoles NAD /min.)	0.0317	0.0216	0.0058	0.0180	0.0215	0.0295
$ZnSO_4$ (mM)	20	2	0.2	10	10	10
ATP (mM)	3.02	3.02	3.02	0.314	0.628	3.02
v(μmoles NAD /min.)	0.0205	0.0128	0.0030	0.0114	0.0146	0.0199
$MnCl_2$ (mM)	20	2	0.2	4	4	4
ATP (mM)	3.02	3.02	3.02	0.157	0.628	3.02
v(μmoles NAD /min.)	*	0.0059	0.0035	0.00333*	0.00436*	0.00594

* precipitation during the enzymic reaction

Table V-4 contd.

	$K_{m(metal)}$ (mM)	$K_{m(ATP)}$ **	$V_{max(metal)}$	$V_{max(ATP)}$ (μmoles NAD/min.)
$MgCl_2$	0.145	0.110	0.0159	0.0141
$NiCl_2$	very high	0.19	very high	0.0223
$CoCl_2$	0.91	0.25	0.0334	0.0313
$ZnSO_4$	1.18	0.28	0.0204	0.0213
$MnCl_2$	0.17	-	0.0065	-

** Total concentrations, uncorrected for buffer effects.

As expected, the maximal velocity approaches approximately the same value in the presence of any particular divalent metal ion whether ATP concentration is varied and metal ion kept at a constant value, or metal ion concentration is varied and ATP concentration held constant. However, there is one exception; with $NiCl_2$ the apparent maximal velocity is vastly different when comparing that obtained by varying $NiCl_2$ concentration ($V_{max(metal)}$) with that when varying ATP concentration ($V_{max(ATP)}$) (Table V-4). This situation could arise if the Ni ions present in the reaction mixture formed a complex with glycylglycine, which is present in a large excess. If this is the case and if the Ni glycylglycine complex has a stability constant comparable with that for the Ni ATP complex, then little Ni ion would be left for forming the latter, especially at the lower

$NiCl_2$ concentrations. Table V-5 lists the stability constants for the complexes of the metal ions concerned here with glycylglycine. The stability constants for the metal ion - ATP complexes are given in Table V-14.

Table V-5

Stability constants for metal ion complexes with glycylglycine.

Metal ion	Glycylglycine - metal ion complex	
	K_1 (mM^{-1})	K_2(mM^{-1})
Mg	0.0115	-
Mn	0.141	-
Co	3.09	0.246
Zn	6.300	0.590
Ni	30.900	2.630

The stability constants noted in Table V-5 are taken from Chem.Soc., London, Special Publication No.6 ('Stability Constants, Part 1 - Organic Ligands', 1957).

Assuming the stability constant for NiATP to be 41.6 mM^{-1} (Table V-14) and neglecting hydrogen ion concentration, it has been calculated for a reaction mixture containing a total of 200 mM glycylglycine, 3 mM ATP and 0.2 mM $NiCl_2$, that at equilibrium the concentrations of Ni glycylglycine and Ni ATP are 0.19999 mM and $< 10^{-5}$ mM respectively. If a total of 20 mM $NiCl_2$ is present, then the concentration of Ni ATP is about 10^{-3}mM. At pH 8.0, less

Ni will be bound to glycylglycine than indicated above, but the general trend will be the same. The calculations in which hydrogen ion concentration is accounted for are rather complicated and will not be attempted here.

These calculations indicate that where a buffer ligand has a stability constant comparable with or higher than that for ATP with respect to their metal complexes, then the concentration of the metal - ATP complex (and even more so, the concentration of free metal) is indeed seriously lowered. If this phenomenon is overlooked, then analysis of the kinetic behaviour of an enzyme system utilizing ATP may lead to false interpretations. Besides the inequalities of the apparent $V_{max(metal)}$ and $V_{max\ (ATP)}$ in the presence of $NiCl_2$, complexing of metal ions with the buffer ligand can explain the finding that the magnitude of $K_{m(metal)}$ increases in the order Mg < Mn < Co < Zn < Ni (Table V-4). This is precisely the order of the magnitude of the stability constants of glycylglycine - metal complexes (Table V-5). Where the metal ion is present in relatively high concentrations, thus tending to nullify the effects due to complexing of metal ions with buffer, as in the case where an apparent K_m for ATP was determined on the presence of each divalent metal ion, the kinetic parameters obtained do not reflect the metal - buffer complex stability constants. In fact the $K_{m(ATP)}$ values obtained (Table V-4) in the presence of each metal do not differ a great deal.

(e) The effect of glycylglycine concentration of the ATP-NMN adenylyltransferase reaction at pH 7.5

To study these effects of the buffer ligand further, a series of experiments were carried out at a lower pH (7.5) and at three different concentrations of glycylglycine (B.D.H.). At each concentration of buffer the $NiCl_2$ concentration was varied between the limits 0.1 mM and 40 mM. Otherwise the conditions of the experiment were the same as described in (d) above. The effect of these variations on the velocity of adenylyl transfer is shown in Table V-6 and graphically in figure V-2. Under the conditions used, 0.0172 µmoles NAD were synthesized per minute when the reaction mixture contained 20 mM-$MgCl_2$, 200 mM-glycylglycine (B.D.H.), pH 7.5, 3.02 mM-ATP and 2.26 mM-NMN (total volume 1.0 ml.).

Table V-6

Rates of ATP:NMN adenylyl transfer at varying levels of glycylglycine in the presence of $NiCl_2$.

200 mM glycylglycine (B.D.H.), pH 7.5							
$NiCl_2$(mM)	40	20	10	4	1.0	0.4	0.1
v(µmoles NAD /min.)	0.0313	0.0365	0.0325	0.0152	0.0037	0.0021	0.0016
100 mM glycylglycine (B.D.H.), pH 7.5							
$NiCl_2$(mM)	40	20	10	4	1.0	0.4	0.1
v(µmoles NAD /min.)	0.0313	0.0366	0.0353	0.0308	0.0118	0.0052	0.0030
50 mM glycylglycine (B.D.H.), pH 7.5							
$NiCl_2$ (mM)	40	20	10	4	1.0	0.4	0.1
v(µmoles NAD /min.)	0.0322	0.0369	0.0358	0.0346	0.0250	0.0118	0.0034

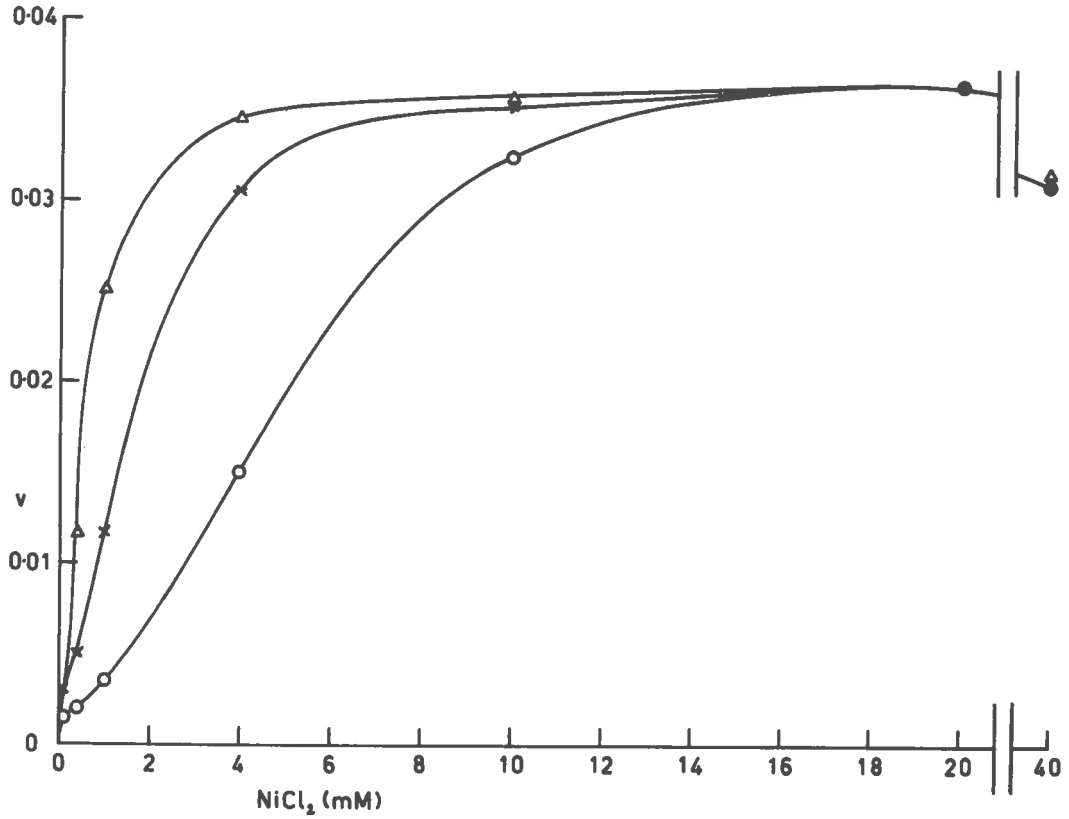
Fig. V-2

Rates of ATP:NMN adenylyl transfer in the presence of $NiCl_2$ at different concentrations of glycylglycine.

○ 200 mM glycylglycine

X 100 mM glycylglycine

△ 50 mM glycylglycine



Adenylyl Transfer in the presence of $NiCl_2$ at different concentrations of glycylglycine

Fig. V-2

A lower pH and lower glycylglycine concentrations should decrease the amount of Ni-glycylglycine complex present in each reaction mixture, and leave more Ni ions available for chelation with ATP. This should increase the rate of NAD synthesis. A comparison of the results compiled in Table V-6 with those in Table V-4 show that at 200 mM-glycylglycine in the presence of 20 mM-$NiCl_2$, the rate was almost doubled in lowering the pH from 8.0 to 7.5. As indicated in Table V-6, at pH 7.5 a lower concentration of glycylglycine also leads to higher rates, especially at $NiCl_2$ concentrations < 10 mM.

(f) Comparison of rates of adenylyl transfer with $NiCl_2$ and $MgCl_2$ in Tris buffers.

In Tris buffers, pH 7.6 and 7.4 (Table V-7), the rates obtained in the presence of 20 mM-$MgCl_2$ are about the same as that obtained in glycylglycine (B.D.H.) buffers. However, 20 and 40 mM-$NiCl_2$ give rates of synthesis higher than was obtained in glycylglycine (B.D.H.) (see Table VI), but lower than that observed in glycylglycine (Sigma) (Table I).

Unlike the result in glycylglycine (B.D.H.), pH 7.5), no inhibition is apparent at 40 mM $NiCl_2$ in Tris buffers. That there is an interaction between Tris (hydroxymethyl) amino methane and Ni ions is apparent, as the higher concentration of buffer leads to lower rates of adenylyl transfer. The reactions were carried out in the presence of 3.02 mM-ATP and 2.26 mM-NMN at the Tris and $MgCl_2$ or $NiCl_2$ concentrations indicated in Table V-7. The reaction

was initiated by the addition of the same transferase preparation used in (d) and (e) above, and the synthesized NAD determined in the customary manner.

Table V-7

Rates of adenylyl transfer in Tris buffers

	150 mM Tris pH 7.6 v(μmoles NAD per min.)	80 mM Tris pH 7.4 v(μmoles NAD per min.)
$MgCl_2$(20 mM)	0.0162	0.0169
$NiCl_2$(20 mM)	0.0324	0.0396
$NiCl_2$(40 mM)	0.0340	0.0458

(g) Inhibition by ethylene diamine tetra acetic acid

If the above effects observed for ATP:NMN adenylyl transfer in the presence of Ni ions are due to chelation of the metal ion with the buffer ligands, thus effectively removing metal ions needed for complexing with the substrate (ATP), then EDTA, a powerful chelating agent, should inhibit the system at certain concentrations. To test this, the transferase was incubated with 3.02 mM-ATP, 200 mM-glycylglycine (B.D.H.), pH 8.0, 2.26 mM-NMN, and either $MgCl_2$ or $NiCl_2$ with or without EDTA (40 mM). The reaction was initiated by the addition of transferase (as in (d), (e) and (f) above), and was carried out at 37° for 10 minutes. After stopping the reaction, the dinucleotide was estimated in the usual manner.

Table V-8 shows that at a concentration of 40 mM, EDTA

completely abolishes all ATP:NMN adenylyltransferase activity in the presence of 4 mM and 0.1 mM-$MgCl_2$, and also at 20 mM-$NiCl_2$. Thus as predicted, EDTA does inhibit the system, and appears to do so in a way that appears qualitatively speaking, to be competitive with the metal ion - 2 mM EDTA suffices to completely inhibit the reaction at 0.1 mM $MgCl_2$, but 40 mM-EDTA only inhibits by 90% at 20 mM-$MgCl_2$. That at 40 mM, EDTA inhibits the activity associated with 20 mM-$MgCl_2$ by 90%, yet at the same concentration completely suppresses adenylyl transfer in the presence of 20 mM-$NiCl_2$, is to be expected from the stability constants of the two metal - EDTA complexes. Log K_1 for the Mg Chelate is reported to be 9.12, while that for the Ni - EDTA is 18.52 (Chem. Soc., London, Special Publication No. 6 'Stability Constants Part 1 - Organic Ligands'. 1957).

Table V-8

Inhibition of ATP:NMN adenylyltransferase by EDTA

Experiment No.	1.	2.	3.	4.	5.	6.	7.	8.	9.
$MgCl_2$ (mM)	20	20	4	4	0.1	0.1	0.1	-	-
$NiCl_2$ (mM)	-	-	-	-	-	-	-	20	20
EDTA (mM)	-	40	-	40	-	40	2	-	40
v(μmoles NAD /min.)	0.0169	0.0017	0.0139	0	0.0061	0	0	0.0187	0
% inhibition	0	90	0	100	0	100	100	0	100

The complete inhibition of the transferase by EDTA, could be applied as a means of stopping the reaction before estimation of dinucleotide. This would be quite feasible as yeast alcohol

dehydrogenase was found to be unaffected by the concentrations of EDTA used at pH 9-10.

(h) Analysis of inhibition by EDTA

The concentrations of the complexes Mg-EDTA and Mg-ATP have been calculated from the known stability constants of these two chelates, for the reaction mixture used above in (g) in which 90% inhibition of the transferase reaction was obtained. Hydrogen ion concentration has been taken into account. The following gives briefly the derivation of the equations necessary to evaluate these concentrations.

In the reaction mixture, before the addition of transferase, there are present the following equilibria:

(i). . . . . $H_2V^{-2} \overset{K_1}{\rightleftharpoons} HV^{-3} + H^+$

(ii) . . . . . $HV^{-3} \overset{K_2}{\rightleftharpoons} V^{-4} + H^+$

(iii) . . . . . $V^{-4} + M^{2+} \overset{K_3}{\rightleftharpoons} MV^{-2}$

(iv) . . . . . $ATP + M^{2+} \overset{K_4}{\rightleftharpoons} MATP$

Here V represents the EDTA molecule, M^{2+} the metal ion, and it will be assumed that V^{-4} is the only EDTA species which binds the metal ion. The ionization of ATP is neglected, and the presence of glycylglycine in the reaction mixture is ignored, as this ligand has a very low affinity for Mg ions compared with EDTA and ATP. If total ATP and $MgCl_2$ present is 3mM and 20 mM respectively, and if at equilibrium the molar concentrations of H_2V^{-2}, HV^{-3}, V^{-4}, MV^{-2}

and MATP are given by a, b, c, x and y respectively, then the concentrations of M^{2+} and ATP at equilibrium are (0.02-x-y) and (0.003-y) molar.

Thus from equations (i) to (iv) above

(1) $K_1 = \frac{Hb}{a}$

(2) $K_2 = \frac{Hc}{b}$

(3) $K_3 = \frac{x}{(0.02-x-y)c}$

(4) $K_4 = \frac{y}{(0.02-x-y)(0.003-y)}$

(5) $c = 0.04-a-b-x$

From (1), $a = \frac{bH}{K_1}$

Substituting in (5), $c = 0.04 - \frac{bH}{K_1} - b - x$

From (2), $b = \frac{cH}{K_2}$

$$\therefore c = 0.04 - \frac{cH^2}{K_1K_2} - \frac{cH}{K_2} - x$$

$$= \frac{0.04 - x}{(1 + \frac{H^2}{K_1K_2} + \frac{H}{K_2})}$$

Substituting in (3), $K_3 = \frac{x}{(0.02-x-y)} \frac{(1 + \frac{H^2}{K_1K_2} + \frac{H}{K_2})}{(0.04 - x)}$ . . . . (6)

Dividing (4) by (6)

$$\frac{K_3}{K_4} = \frac{x(1 + \frac{H^2}{K_1K_2} + \frac{H}{K_2})}{(0.04 - x)} \frac{(0.003-y)}{y}$$

$$\therefore x = \frac{K_3y(0.04)}{K_3y + K_4(0.003-y)(1+ \frac{H^2}{K_1K_2} + \frac{H}{K_2})}$$

Substituting in (5), and denoting $(1 + \frac{H^2}{K_1K_2} + \frac{H}{K_2})$ by D then

$$K_4 = \frac{K_3y^2 + K_4y(0.003-y)D}{[(0.02)K_3y + (0.02)K_4(0.003-y)D - K_3y^2 - K_4y(0.003-y)D - K_3y(0.04)][0.003-y]}$$

which in another form is

$$y^3K_4[K_3 - K_4D]$$
$$+ y^2[2K_4^2(0.003)D + K_3K_4(0.04) - K_3K_4(0.003) - K_3K_4(0.02) - K_3K_4D + K_4^2(0.02)D]$$
$$+ y\,K_4(0.003)[K_3(0.02) + D - 2K_4(0.02)D - K_4(0.003)D - K_3(0.04)]$$
$$+ K_4^2(0.003)^2(0.02)D$$
$$= 0 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

Hence the solution of equation (7) gives the concentration of the Mg-ATP complex (y), given the values of K_1, K_2, K_3 and K_4 and the pH of the reaction mixture involved. The stability constants have been recorded as $K_1 = 6.3 \times 10^{-7}$M; $K_2 = 5.5 \times 10^{-11}$M; $K_3 = 1 \times 10^{9}M^{-1}$ (Chem. Soc., London, Special Publication No.6, "Stability Constants Part 1 - Organic Ligands", 1957); $K_4 = 2.45 \times 10^{4}M^{-1}$ (Nanninga, 1961). At pH 8.0, $H = 1 \times 10^{-8}$M.

Under these conditions, equation (7) becomes

$$y^3(2.4389 \times 10^{13}) + y^2(4.18393 \times 10^{11}) - y(1.48437 \times 10^{9}) + 2.006 \times 10^{4} = 0$$

From this it was deduced that $y = 1.3 \times 10^{-5}$M (Mg-ATP)

and so $x = 1.998682 \times 10^{-2}$M (Mg-EDTA)

and $Mg^{2+} = 1.78 \times 10^{-7}$M (free Mg^{2+})

Part of the latter will, of course, be present as the glycylglycine complex. These concentrations apply to the conditions for experiment number two in Table V-8. The control (experiment No.1, Table V-8), in which no EDTA was present is rather similar to one of the reaction mixtures described in Table IV-3, where the concentrations of MgATP, Mg glycylglycine, free Mg and free ATP were calculated to be 3.125, 8.372, 8.503 and 0.015 mM respectively.

Now knowing the concentrations of the Mg-ATP chelate present in experiments 1 and 2 (Table V-8), and knowing the rate of adenylyl transfer in experiment 1, where no EDTA was present, one can predict the rate of NAD formation in experiemnt 2, where EDTA was added to a total concentration of 40mM. A K_m for Mg ATP of 0.15 mM (see section IV) is assumed.

For experiment 1, where the concentration of Mg-ATP is approximately 3.0 mM, 0.0169 µmoles NAD were synthesized per minute. Using the Michaelis equation,

$$v = \frac{V_{max}}{1 + \frac{K_m}{MgATP}}$$

a V_{max} of 0.01775 µmoles NAD/min was determined for the enzymic reaction. For experiment 2, the concentration of Mg-ATP was calculated to be 0.013 mM. Using the above V_{max}, the Michaelis equation predicts a rate of 0.0015 µmoles NAD synthesized/minute. This is very close to the observed velocity (0.0017 µmoles NAD/minute), and indicates that this type of problem can be tackled on the basis of the MgATP complex being the actual substrate species utilized by the

transferase. However, it must be emphasized here, that experimentally it has not been determined if EDTA has any effects on the transferase protein itself; such an interaction could effect the rate of NAD synthesis.

(i) Kinetic parameters for ATP in the presence of Mg, Ni and Co ions.

It would appear from the present work that the buffer ligands interfere seriously with the rates of adenylyl transfer, especially where Ni ions are present, due to competition with ATP for the divalent metal ions. This effect is of course more marked at low concentrations of metal ion, and makes the interpretation of the kinetic behaviour obtained by varying metal ion concentration rather complicated. However, if the metal ion is kept at a relatively high and constant concentration, and ATP concentration is varied, these effects are minimized. The kinetic parameters so obtained could tell us more about the nature of the metal ion specificity.

To this end the transferase was incubated at 37° with reaction mixtures containing 100 mM-glycylglycine (B.D.H.), pH 7.5, 2.26 mM-NMN and 20mM-$MgCl_2$, $NiCl_2$ or $CoCl_2$. ATP concentration was varied from 0.157 mM to 7.55 mM. The reaction was initiated by 0.05 ml. of a transferase preparation containing 45 γ of protein (specific activity 0.5 μmoles NAD per min. per mg. of protein) as in (d), (e), (f) and (g) above. After 10 minutes (in the presence of $MgCl_2$) or five minutes ($NiCl_2$ and $CoCl_2$), the reaction was stopped and the estimation of NAD was carried out as before.

Table V-9 and figure V-3 show the rates obtained under these conditions; they conform to the usual Michaelis Menten behaviour. As indicated in Table V-9, the apparent K_m for ATP (or the metal-ATP complex) has very nearly the same value whether Mg, Ni or Co ions are present in the reaction mixtures. However, a higher V_{max} is obtained in the presence of Ni or Co ions compared with that found when Mg ions are a component of the reaction.

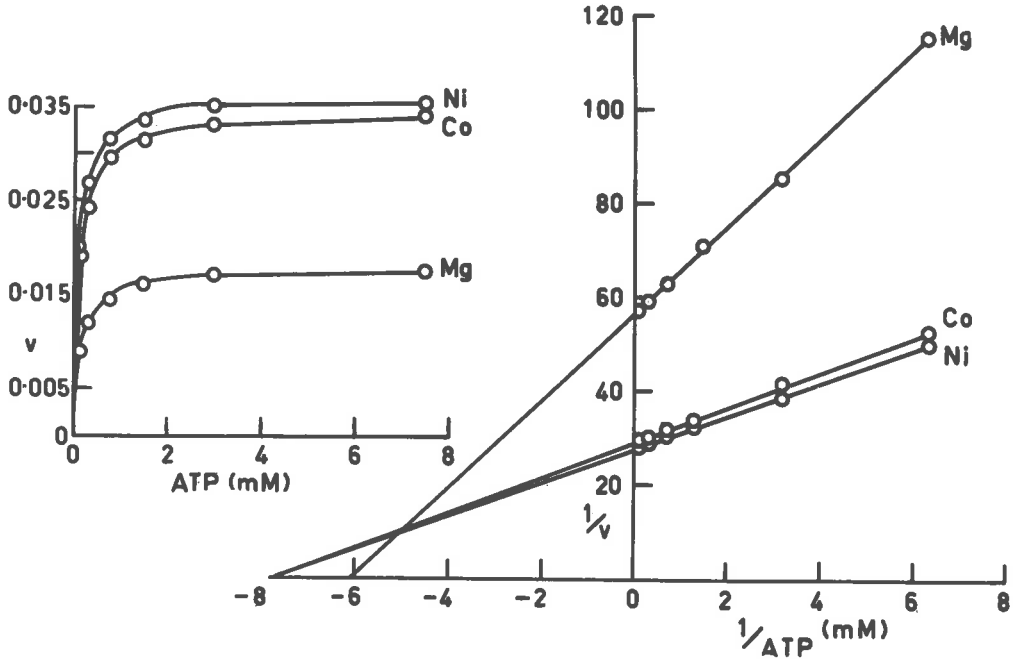
(j) The effect of $BeCl_2$ on the ATP:NMN adenylyltransferase reaction.

It was found that $BeCl_2$ (20mM or 4mM) in the presence of 4.52 mM-NMN, 4 mM-ATP, 200 mM-glycylglycine (Sigma) pH 7.6 and ATP:NMN adenylyltransferase (total volume 1.0 ml.) did not give rise to any detectable amounts of NAD. A control, containing 16 mM-$MgCl_2$ in place of $BeCl_2$, was found to have synthesized 0.18 µmoles of NAD under the same conditions. The smallest detectable amount of NAD under the conditions of the experiment would have been approximately 1% of this figure. A slight precipitate was observed to have formed in the cases where Be^{2+} ions were present.

To test $BeCl_2$ as an inhibitor, the transferase was incubated with reaction mixtures containing 4.52 mM-NMN, 4 mM-ATP, 200 mM-glycylglycine (Sigma), pH 7.6 and either $MgCl_2$ or $NiCl_2$, with or without $BeCl_2$. Where $MgCl_2$ was present, addition of $BeCl_2$ gave rise to a slight precipitate. No precipitate was formed when $BeCl_2$ was added to the reaction mixture containing $NiCl_2$. The enzymic reaction was started by the addition of 0.1 ml. of a partly purified

Fig. V-3

Effect of ATP concentration on adenylyl transfer in the presence of $NiCl_2$, $CoCl_2$ and $MgCl_2$.



Effect of ATP Concentration on ATP:NMN Adenylyl Transfer in presence of $NiCl_2$, $CoCl_2$ and $MgCl_2$

Fig. V - 3

Table V-9

The effect of ATP concentration on adenylyl transfer in the presence of $MgCl_2$, $NiCl_2$ or $CoCl_2$.

$MgCl_2$ 20mM						
ATP(mM)	0.157	0.314	0.785	1.51	3.02	7.55
$^1/_{ATP}$	6.36	3.18	1.274	0.662	0.331	0.1325
v(μmoles NAD per min.)	0.0087	0.0119	0.0144	0.0161	0.0170	0.0174
$^1/_v$	115.0	84.0	69.5	62.1	58.9	57.5
$NiCl_2$ 20mM						
ATP(mM)	0.157	0.314	0.785	1.51	3.02	7.55
$^1/_{ATP}$	6.36	3.18	1.274	0.662	0.331	0.1325
v(μmoles NAD per min.)	0.0199	0.0267	0.0315	0.0336	0.0350	0.0352
$^1/_v$	50.2	37.4	31.8	29.8	28.6	28.4
$CoCl_2$ 20mM						
ATP(mM)	0.157	0.314	0.785	1.51	3.02	7.55
$^1/_{ATP}$	6.36	3.18	1.274	0.662	0.331	0.1325
v(μmoles NAD per min.)	0.0190	0.0240	0.0295	0.0313	0.0331	0.0346
$^1/_v$	52.6	41.65	33.9	32.0	30.0	28.9

Metal ion	Mg^{2+}	Co^{2+}	Ni^{2+}	
$K_{m(ATP)}$	0.16mM	0.13mM	0.13mM	
$V_{max(ATP)}$	0.0179	0.0342	0.0367	(μmoles NAD per min.)

ATP:NMN adenylyltransferase preparation containing 9 γ of protein (specific activity 1.28 μmoles NAD synthesized per minute per mg. of protein). After 10 minutes at 37° the reaction was stopped. The rates of adenylyl transfer were calculated from the amount of NAD formed in each case.

As shown in Table V-10, $BeCl_2$ inhibits adenylyltransfer in the presence of either $NiCl_2$ or $MgCl_2$.

Table V-10

Inhibition of adenylyl transfer by $BeCl_2$

$MgCl_2$(mM)	16	16	-	-
$NiCl_2$(mM)	-	-	20	20
$BeCl_2$(mM)	-	4	-	4
v(μmoles NAD per min.)	0.00911	0.00698	0,0280	0.0008
Relative activity	100.0	76.6	307.0	8.8

(k) Effect of NaF on ATP:NMN adenylyltransferase in the presence of various divalent metal ions.

The transferase was incubated with reaction mixtures containing 100 mM-glycylglycine (B.D.H.), pH 7.5, 2.26 mM-NMN, 3.02 mM-ATP and either 2 mM-$MgCl_2$, $NiCl_2$, $ZnSO_4$, $MnCl_2$ or $CoCl_2$ with or without 50 mM-NaF. No precipitations were observed at these bw cation concentrations. The reaction was started by the addition of 0.05 ml. of a transferase preparation containing 45 γ of protein (specific activity 0.5 μmoles NAD synthesized per min. per mg.), and stopped after five minutes

where $NiCl_2$ or $CoCl_2$ was present, or ten minutes where $MgCl_2$, $ZnSO_4$ or $MnCl_2$ was included in the reaction. The dinucleotide formed in each case was estimated in the usual manner.

Table V-11 shows that addition of NaF caused no significant effect on the rate of NAD formation under the conditions used with reaction mixtures containing either Mg, Co, Ni, Mn or Zn ions.

Table V-11

Effect of NaF on the ATP:NMN adenylyltransferase reaction.

	$MgCl_2$		$NiCl_2$		$CoCl_2$		$ZnSO_4$		$MnCl_2$	
NaF (mM)	0	50	0	50	0	50	0	50	0	50
v(μmoles NAD per min.)	0.0136	0.0143	0.0183	0.0174	0.0262	0.0268	0.0216	0.0226	0.0053	0.0047

D. Discussion

Metal ion specificity of the pyrophosphorylases and the effect of complexing ligands

The metal ion specificity of ATP:NMN adenylyltransferase has been examined by Dr. M. R. Atkinson (unpublished results). He has surveyed a much wider range of divalent metal ions than that described above, using a coupled assay system to detect adenylyl transfer. The reactions were carried out directly in spectrophotometer cells containing 3 mM-NMN, 3mM-ATP, 100 mM-Tris (sulphate), pH 8.0, 0.3 M-ethanol, ATP:NMN adenylyltransferase and an excess of yeast alcohol dehydrogenase. The reaction, if any, was inititated by the addition of a metal ion solution, to give a final concentration of 0.2 mM with respect to the divalent cation. The formation of reduced NAD was followed by the increase in optical density at 340 mμ. Table V-12 gives the rate of NADH formation obtained in the presence of each divalent cation relative to that obtained with $MgSO_4$.

It would seem from Table V-12 and the experiments described in the results section, that the cations supporting adenylyl transfer are Ni^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} and Cd^{2+}, and that Cu^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} and Be^{2+} do not assist in the enzymic transfer reaction. In addition to these, Fe^{2+} was also tried (as $FeSO_4$) by Dr. Atkinson, but the results were uncertain. With this cation an increase in optical density in the region of 340 mμ was observed in his assay system in the absence of the transferase. This point needs further investigation.

Table V-12

Metal ion specificity of ATP:NMN adenylyltransferase.

Metal salt (0.2mM)	Relative rate
$MgSO_4$	1.00
$MnCl_2$	0.39
$CoCl_2$	1.29
$NiSO_4$	0.48
$ZnSO_4$	0.88
$CdSO_4$	0.03
$CaCl_2$	0
$SrCl_2$	0
$BaCl_2$	0
$CuSO_4$	0

Any kinetic work carried out in this system, in which the aim is to compare the rates of adenylyl transfer in the presence of various divalent cations, is necessarily complicated because of the presence of buffer ions which interact with these metals. The buffer molecules are present in a large excess and will have a different affinity for each of the metals concerned, thus disguising effects due to enzymic transfer. Fortunately stability constants are available in the case of the glycylglycine ligand, (Table V-5), so that it is possible to make allowances for this. It was for this reason that the present work was carried out in glycylglycine buffer. systems, in the hope that accurate corrections could be made.

However, the purity of the buffer used is obviously suspect, when one compares the results found in glycylglycine (Sigma) (Table V-1) with those in glycylglycine (B.D.H.) (Table V-6 and Table V-9), although the reactions were carried out at different NMN concentrations and with different ATP:NMN adenylyltransferase preparations. In addition the calculations necessary to evaluate these corrections are extremely complicated, especially as it is essential to take hydrogen ion concentrations into account.

Tris is a potentially powerful chelating agent, possessing three hydroxyl groups and an amino group, and has the added disadvantage that no stability constants are available for its metal-chelate complexes which could be applied in the present work. The only studies describing the nature and extent of actual complex formation by Tris have been performed with Ag^+ as the metal ion. Benesch and Benesch (1955) found log K_1 = 3.09 and log K_2 = 3.47 for the stepwise dissociation constants for Ag^+ and one or two moles of Tris respectively. In comparison, glycylglycine and Ag^+ show log K_1 = 2.72 and log K_2 = 2.26 (Monks, 1951). N-Ethylmorpholine might be suitable for this purpose.

Because of these complications due to buffer ions, the only way in which a kinetic comparison of the behaviour of these divalent cations could be carried out in the present system would be to omit buffer ions altogether, and use reagents carefully titrated to the appropriate pH before addition of the transferase. It has been postulated that there should be an increase in pH during the reaction in which pyrophosphoate is formed following adenylyl transfer, due to the high pK_a' (9.5) of the fourth dissociation of pyrophosphate

(Imsande and Handler, 1961). If this is so, this type of experiment would not easily be carried out. However, it is felt that the divalent cations present would suppress this dissociation almost entirely and no significant pH changes have been found in large scale experiments with a pH stat.

Because of these buffer effects, it is difficult to sum up the results obtained by rate studies. However, consideration of Tables V-4 and V-11 suggest that the maximum velocity obtained in the presence of each metal ion is different and follows the order $Ni^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Mn^{2+} > Cd^{2+}$. The K_m for ATP appears to be unchanged whatever cation is used, at least in the case of Mg^{2+}, Co^{2+} and Ni^{2+} (Table V-9). These results would follow from a mechanism in which the metal plays little part in the binding of ATP to the enzyme, but is essential for the adenylyl transfer reaction at the active centre. It would also follow from this that ATP (uncomplexed) should act as a competitive inhibitor, if the enzyme has little affinity for the cation itself. This phenomenon has been described in Section IV.

A divalent metal ion seems to be necessary for the reactions catalysed by other pyrophosphorylases which, like ATP:NMN adenylyltransferase, synthesize a pyrophosphate diester from a nucleoside triphosphate and an orthophosphate ester of an alcohol, liberating inorganic pyrophosphate. Table V-13 shows the known metal ion specificity of the 24 pyrophosphorylases so far described. Some of these enzymes have not been extensively purified (CTP:choline phosphate cytidylyltransferase, CTP:ethanolaminephosphate cytidylyltransferase, UTP:glucuronic acid phosphate uridylyltransferase, UTP:galacturonic

acid phosphate uridylyltransferase, UTP:xylose phosphate uridylyltransferase, UTP:arabinose phosphate uridylyltransferase). Others have only very recently been described and their properties only briefly reported (ATP:glucose phosphate adenylyltransferase, CTP:glucose phosphate cytidylyltransferase, GTP:D-glycero D mannoheptose phosphate guanylyltransferase, dTTP:galactose phosphate thymidylyltransferase). At least one, UTP:glucosamine phosphate uridylyltransferase, which so far has only been demonstrated in crude systems (Maley, Maley and Lardy, 1956; Maley and Maley , 1959; Maley and Lardy, 1956; Silbert and Brown, 1961) may be the result of the activity of another pyrophosphorylase, as Maley and Garrahan, (1960) have shown that highly purified UTP: glucosephosphate uridylyltransferase can synthesize UDP glucosamine from UTP and glucosamine phosphate.

However, despite these limitations, many of the pyrophosphorylases have already been shown to have quite a broad specificity with respect to the metal ion, and it may be that they all will eventually be shown to have similar requirements to that exhibited by ATP:NMN adenylyltransferase. As indicated in Table V-13, 21 have been shown to use Mg^{2+}, 9 Mn^{2+}, 2 Ni^{2+}, 7 Co^{2+} and only ATP:NMN adenylyltransferase has so far been shown to be capable of using Zn^{2+} as the metal cofactor. In no case has Ca^{2+} been shown to be effective in satisfying the divalent ion requirement.

If the chelation of metal ions by buffer ligands renders the metal ion unavailable for participation in adenylyl transfer as suggested above, then more powerful complexing agents, such as EDTA, should give an even more pronounced effect. This has been shown to be

Table V-13

Pyrophosphorylase	Mg^{2+}	Mn^{2+}	Ni^{2+}	Co^{2+}	Zn^{2+}	Reference
ATP:NMN adenylyltransferase	+	+	+	+	+	Kornberg (1950); present investigations
ATP:nicotinic acid mononucleotide adenylyltransferase	+	.	.	.	.	Imsande (1961)
ATP:FMN adenylyltransferase	+	+	.	.	.	Schrecker & Kornberg (1950)
ATP:4'-phosphopantetheine adenylyltransferase	+	.	.	.	.	Hoagland & Novelli (1954)
ATP:glucose-1-phosphate adenylyltransferase	.	.	.	.	.	Recondo & Leloir (1961)
CTP:choline phosphate cytidylyltransferase	+	+	.	.	.	Borkenhagen & Kennedy (1957)
CTP:ethanolamine phosphate cytidylyltransferase	+	.	.	.	.	Kennedy & Weiss (1956)
CTP:glycerol phosphate cytidylyltransferase	+	+	.	+	.	Shaw (1962)
CTP:ribitol phosphate cytidylyltransferase	+	.	.	.	.	Shaw (1962)
CTP:glucose phosphate cytidylyltransferase	+	.	.	.	.	Ginsberg, O'Brien & Hall (1962a)
UTP:glucose phosphate uridylyltransferase	+	+	+	+	.	Turner & Turner (1958)
UTP:galactose phosphate uridylyltransferase	+	+	.	+	.	Neufeld et al., (1957)

Table V-13 contd.

UTP:Nacetylglucosamine phosphate uridylyltransferase	+	+	.	+	.	Pattabiraman & Bachhawat (1961).
UTP:Nacetylgalactosamine phosphate uridylyltransferase	+	.	.	.	.	Maley, Maley & Lardy (1956)
UTP:glucosamine phosphate uridylyltransferase	+	.	.	.	.	Maley, Maley & Lardy (1956)
UTP:glucuronic acid phosphate uridylyltransferase	+	.	.	.	.	Feingold, Neufeld & Hassid (1958)
UTP:galacturonic acid phosphate uridylyltransferase	+	.	.	.	.	Feingold, Neufeld & Hassid (1958)
UTP:xylose phosphate uridylyltransferase	+	+	.	+	.	Neufeld et al. (1957)
UTP:arabinose phosphate uridylyltransferase	+	+	.	+	.	Neufeld et al.(1957)
GTP:mannose phosphate guanylyltransferase	+	.	.	.	.	Munch-Petersen (1956)
GTP:glyceromannoheptose phosphate guanylyltransferase	.	.	.	.	.	Ginsberg, O'Brien & Hall (1962)
GTP:glucose phosphate guanylyltransferase	+	.	.	.	.	Carlson & Hansen (1962)
dTTP:glucose phosphate thymidylyltransferase	+	.	.	.	.	Kornfeld & Glaser (1961)
dTTP:galactose phosphate thymidylyltransferase	.	.	.	.	.	Fazur, Kleppe & Cepure (1962)

the case (Table V-8). Although analysis of the inhibition due to EDTA would suggest the above mechanism (Results, section h), it has not been proven experimentally that EDTA has no other effects on the transferase.

Three other pyrophosphorylases have been examined briefly for inhibition by the ligand. Munch-Petersen (1955) reports that the reaction catalysed by UTP:glucosephosphate uridylyltransferase is not affected by 10 mM-EDTA in the presence of $MgCl_2$. However, the concentration of $MgCl_2$ is not indicated, and the reaction was carried out at pH 7.2, where EDTA has less affinity for metal ions than at the hydrogen ion concentration employed in the present work (pH 8.0).

The UTP:galactose phosphate uridylyltransferase reaction has been shown to be inhibited completely by 10 mM-EDTA in the presence of 10 mM-$MgCl_2$ at pH 7.6 (Isselbacher, 1958). This is completely in agreement with the present investigations.

At pH 7.4, Pattabiraman and Bachhawat (1961) found that UTP:N-acetylglucosamine phosphate uridylyltransferase activity was inhibited 60% by 10 mM-EDTA. This inhibition was reversed by the addition of 20 mM-$MgCl_2$. They do not indicate the initial metal ion concentration and the system is complicated by the fact that these investigators found transferase activity without addition of metal ions. The latter is probably explained by the fact that the enzyme used had only been purified 46 fold from the initial sheep brain extract.

The effects of EDTA on other pyrophosphorylases of the same type have not been described as yet.

Inhibition of ATP:NMN adenylyltransferase by $BeCl_2$

The investigations into the effect of this compound on ATP:NMN adenylyltransferase were complicated by precipitations in the reaction mixture. However, in the presence of 20 mM-$NiCl_2$ and at 4.52 mM-NMN, 4 mM-ATP, pH 7.6, no precipitation occurred on the addition of $BeCl_2$ to a concentration of 4 mM. It is significant that in this instance a 97% inhibition was observed, whereas where 16 mM-$MgCl_2$ was used, a precipitation occurred on adding $BeCl_2$, and only a 23.4% inhibition resulted (Table V-10).

Stirpe and Aldridge (1961) found that intravenous injection into rats of 0.6 mg. $BeSO_4$/Kg body weight gave a significant lowering (by 25%) of the specific activity of ATP:NMN adenylyltransferase contained in the nuclei of rat liver cells, isolated 24 hours after treatment. This may suggest that beryllium ions are capable of binding to the enzyme in some way and blocking adenylyl transfer at the active centre. It has been shown that Be^{2+} has a very high affinity for ATP (see Table V-14). The mechanism by which this metal ion inhibits the transferase is uncertain as yet and is discussed further below.

Other enzymes are known to be inactivated by this ion (e.g. carnosinase - Rosenberg, 1960). Alkaline phosphatase has been investigated rather thoroughly from this point of view (Morton, 1955; Ahmed and King, 1960). Klemperer, Miller and Hill (1949) claim that the inhibition of alkaline phosphatase by Be^{2+} is independent of the

nature of the substrate. The inhibition by Be^{2+} was found to be reversed to some extent by Mg^{2+} (Aldridge, 1950; see also Morton, 1955) It has been shown that aurine tricarboxylic acid reversed the inhibition (Lindenbaum, White and Schubert, 1954), and it has been suggested that the effectiveness of this ligand can be accounted for by bringing about reversible chelation of Be^{2+} with alkaline phosphatase (Schubert and Lindenbaum, 1954).

It is of interest to note here that Be^{2+} is the most active of the divalent metal ions necessary for the non-enzymic activation of acetate by ATP (Lowenstein and Schatz, 1961).

Effect of sodium fluoride on ATP:NMN adenylyltransferase

Fluoride ion is inhibitory to many enzymes acting on phosphate esters, including some inorganic pyrophosphatases (Elliot, 1957). In the case of phosphoglucomutase this inhibition appears to be due to the formation of a Mg-fluorophosphate complex (Najjar, 1948). The present work was prompted by the investigations of Strominger and Smith (1959), who found that the pyrophosphorolysis of UDP-Nacetyl-glucosamine by UTP:Nacetylglucosamine phosphate uridylyltransferase from Staphylococcus aureus was inhibited by 10 mM-NaF when Mg^{2+} was the activating ion. No inhibition was found when Mn^{2+} was used in place of Mg^{2+}.

Sodium fluoride (50 mM) was not found to be inhibitory to ATP:NMN adenylyltransferase, when Ni^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+} or Mn^{2+} was used as the metal cofactor. Kornberg (1950) had already shown that 50 mM-fluoride does not inhibit this transferase in the presence of Mg^{2+}.

Other pyrophosphorylases which have been shown not to be affected by fluoride in the presence of Mg^{2+} are CTP:cholinephosphate cytidylyltransferase (Borkenhagen and Kennedy, 1957), CTP:glycerophosphate cytidylyltransferase (Shaw, 1962), UTP:glucosephosphate uridylyltransferase (Munch-Petersen, 1955) and calf liver UTP:Nacetylglucosaminephosphate uridylyltransferase (Strominger and Smith, 1959). It is interesting that the latter enzyme behaves differently to fluoride, depending whether it is prepared from calf liver or Staphylococcus aureus. There is no information available for the other pyrophosphorylases relating to the effects of fluoride ion.

General considerations of metal ion specificity

The metal ion specificity of enzymes transferring phosphate groups exhibits a bewildering pattern. If generalisations have to be made, it would seem that most enzymes transferring $-PO_3^{2-}$ groups are at least activated by some divalent metal ions, and that kinases, which utilize a nucleoside triphosphate, require divalent cations for reaction. Magnesium and manganese are encountered most often here (Koshland, 1959). As indicated in Table V-13, the pyrophosphorylases, as far as is known, require divalent cations for nucleosidyl transfer.

If the only prerequisite for the metal ion in promoting the transfer of phosphate groups from nucleoside triphosphates is its ability to form a chelate with this type of polyphosphate, then a wide range of divalent cations have to be considered as likely to aid the reaction. The stability constants of various metal-ATP^{2-} complexes are listed in Table V-14.

Table V-14

-log stability constants of metal-ATP^{2-} complexes (M^{-1}).

Metal ion	T. and M.*	B. and F.*	N.*	S. and A.*	W.*
Cu^{2+}	6.13	5.50	-	-	-
Be^{2+}	-	5.01	-	-	-
Cd^{2+}	-	4.70	-	-	-
Ni^{2+}	5.02	4.61	-	-	-
Zn^{2+}	4.85	-	-	-	-
Mn^{2+}	4.78	-	-	3.98	4.75
Co^{2+}	4.66	4.53	-	-	4.62
Mg^{2+}	4.22	-	4.39	3.47	4.04
Ca^{2+}	3.97	-	3.93	3.29	3.77
Sr^{2+}	3.54	-	3.60	3.03	-
Ba^{2+}	3.29	-	3.36	-	-

*T. and M. from Taqui Khan and Martell (1962)

B. and F. Brintzinger and Fallab (1960)

N. Nanninga (1961)

S. and A. Smith and Alberty (1956)

W. Walaas (1958)

The univalent alkali metal ions form only weak complexes with ATP, and the tervalent cations have not been investigated for their ability to interact with this polyphosphate.

It is noteworthy that all metal ions active in ATP:NMN adenylyl transfer (Ni, Co, Zn, Mg, Mn and Cd) form complexes with high stability constants with ATP. However this cannot be the only factor involved, as it has been shown that Cu^{2+}, Be^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}, all able to form complexes with ATP, are not active in adenylyl-transfer. In addition, the order of maximum velocities obtained with each metal ion (Ni > Co > Zn > Mg > Mn > Cd) does not follow the order of numeral values of stability constants with ATP (Cd > Ni > Zn > Mn > Co > Mg). As far as other enzymes are concerned, hexokinase, when catalysing the phosphorylation of fructose with ATP, has been shown to use the same six metal ions found active in adenylyl transfer and again Ca^{2+}, Cu^{2+} and Be^{2+} do not aid the reaction at all (Brintzinger and Fallab, 1960). On the other hand, another kinase, muscle phosphoglycerate kinase, can use Mg^{2+} or Mn^{2+} ions, but not Zn^{2+}, Co^{2+} or Ni^{2+} (Rao and Oesper, 1961). Clearly in these three enzyme systems, other factors are involved besides the necessary condition that the metal ions form a complex with ATP. It is likely that these factors have to be considered separately for each enzyme, perhaps depending on the nature of its active site and the exact mechanism of the reaction it catalyses.

Even in reactions involving ATP, where no enzyme is required, the metal ion requirements are by no means easily explained. Lowenstein (1958) showed that the non-enzymic transfer reaction involving ATP and inorganic phosphate (equation 1)

$$(1)\quad \text{ATP} + \text{inorganic orthophosphate} \longleftrightarrow \text{ADP} + \text{inorganic pyrophosphate}$$

required certain divalent ions, and had a pH optimum at 9.0. The magnitude of the rate of reaction obtained with each metal ion was in the order $Ca^{2+} > Mn^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+}$ and Fe^{2+}, Cu^{2+}, Ni^{2+}, Be^{2+} and Mg^{2+} gave little or no reaction.

Yet another non-enzymic reaction, apparently related to the above, showed an altogether different metal ion specificity (Lowenstein and Schatz, 1961). When acetate is used as an acceptor in place of inorganic phosphate, and the product trapped as the hydroxamate (equation 2)

$$(2)\quad CH_3-\overset{O}{\overset{\|}{C}}-O^- + ATP + NH_2OH \longrightarrow CH_3-\overset{O}{\overset{\|}{C}}-\overset{H}{\overset{|}{N}}-OH + ADP + \text{inorganic ortho-phosphate}$$

the reactivity of the divalent ions was in the order $Be^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+}$. The ions Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+} and Hg^{2+} gave no reaction at all, and no reaction occurred in the absence of divalent ions. The results with Cu^{2+} were reported to be erratic, and no satisfactory comparison with the other cations was possible. The pH optimum of the transfer was 5.2. The differences in metal specificity in reactions (1) and (2) must lie in differences of reaction mechanism. It is noteable that the order of reactivity of the metal ions (with the exception of Be^{2+}) in reaction (2), is not unlike that obtained for enzymic ATP:NMN adenylyl transfer.

Many investigators have attempted to relate the activities of various divalent ions in enzymic systems with the ionic radii (crystal) of the ions concerned, and have achieved some correlation in many cases (see Malmstrom and Rosenberg, 1959; Dixon and Webb, 1958, p.448; Williams, 1959).

A correlation between the velocities obtained for each cation in ATP:NMN adenylyl transfer and crystal ionic radii is shown in figure V-4 (a). The rate data (V_{max}) for Mg^{2+}, Co^{2+} and Ni^{2+} are taken from Table V-9, the rate for Mn^{2+} was taken as being 30% of that for Mg^{2+} (Tables V-1, V-4, V-11 and V-12) and for Cd^{2+} 3% of the figure for Mg^{2+} (Table V-12). Zn^{2+}, for which there are no reliable data, was taken as having a V_{max} between that of Co^{2+} and Mg^{2+}, as indicated in Tables V-4 and V-11. It is realized that in some cases rates obtained in the presence of Zn^{2+} were lower than that obtained with Mg^{2+} (as in Tables V-1 and V-12) however under the circumstances it is reasonable to assume the lower values to be due either to interaction with buffer ligands (Table V-12) or precipitation (Table V-1).

The plot of ionic radius against maximal velocity for each divalent cation gives a bell-shaped curve, suggesting that divalent cations with an ionic radius of about 0.70 Å are most active in promoting adenylyl transfer, and that ions with radii smaller or larger than this have less or no activity. The curve predicts that Be^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} will have no activity. However, Cu^{2+}, which falls in the active range, has been shown to be inactive in adenylyl transfer (Table V-12).

It has been pointed out by Williams (1954) that such properties as ionic radii, ionization potentials, electronegativities and entropies of hydration of a group of cations show some mutual interdependence. With this in view, and since the heats of hydration of a wide range of cations have been evaluated, we have plotted this property against

Fig. V-4

The relationship between rate of ATP:NMN adenylyl transfer and ionic radius, heat of hydration and entropy of hydration for the various activating divalent metal ions.

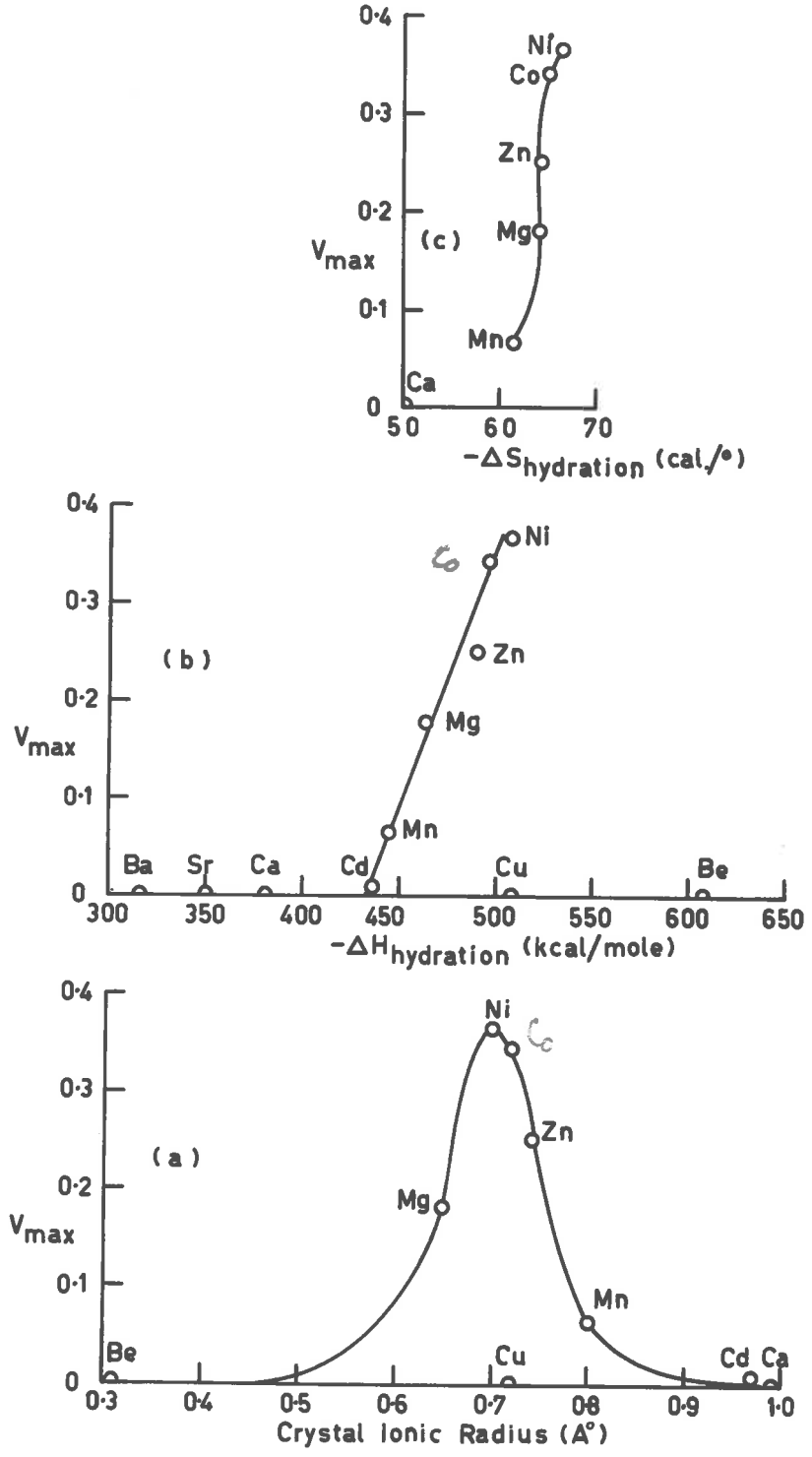

0·4
0·3
0·2
0·1
0
Vmax
(c)
Ni
Co
Zn
Mg
Mn
Ca
50
60
70
-ΔShydration (cal./°)
(b)
Ba
Sr
Ca
Cd
Cu
Be
300
350
400
450
500
550
600
650
-ΔHhydration (kcal/mole)
(a)
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Crystal Ionic Radius (A°)

Fig. V - 4

velocities of adenylyl transfer. Figure V-4(b) shows that an almost linear relationship is obtained, and again Ca^{2+}, Sr^{2+} and Ba^{2+} are excluded from promoting transferase activity. Cu^{2+} and Be^{2+} do not fit the relationship. Heats of hydration were taken from Williams (1954), except that for Be^{2+}, which is recorded by Conway and Bockris (1954). The data obtained by Lowenstein and Schatz (1961) for the relative activities of divalent cations in carrying out reactions (1) and (2) above, also appear to fit a $-\Delta H_{hydration}$ versus activity plot such as this (figure V-5). The relationship for reaction (2) is very similar to that for adenylyl transfer to NMN, except that Be^{2+} gives a high rate of transfer in the former. As expected, there is a correlation between a closely related property, the entropy of hydration of cations and the rate of adenylyl transfer (figure V-4(c)). The value for entropy of hydration for each cation was taken from Nancollas (1961).

It is not clear at present whether it is the size of the ion which is important in promoting ATP:NMN adenylyl transfer, or some other property closely connected with it. However, it is the change in enthalpy of a reaction which has to be taken as a measure of the strength of the chamical bonds which are broken or formed during the reaction. This may be important if the divalent cation is considered to act mainly in inducing adenylyl transfer rather than playing the more passive role of binding ATP to the active centre. From this point of view it is noteworthy that the heat of hydration of an ion can be correlated with its coefficient of polarization (Guerdjckov, 1961). This property, also termed polarizability, is a measure of the ease

Fig. V-5

The relationship between the rate of reactions (1) and (2) and the heat of hydration of various activating divalent metal ions.

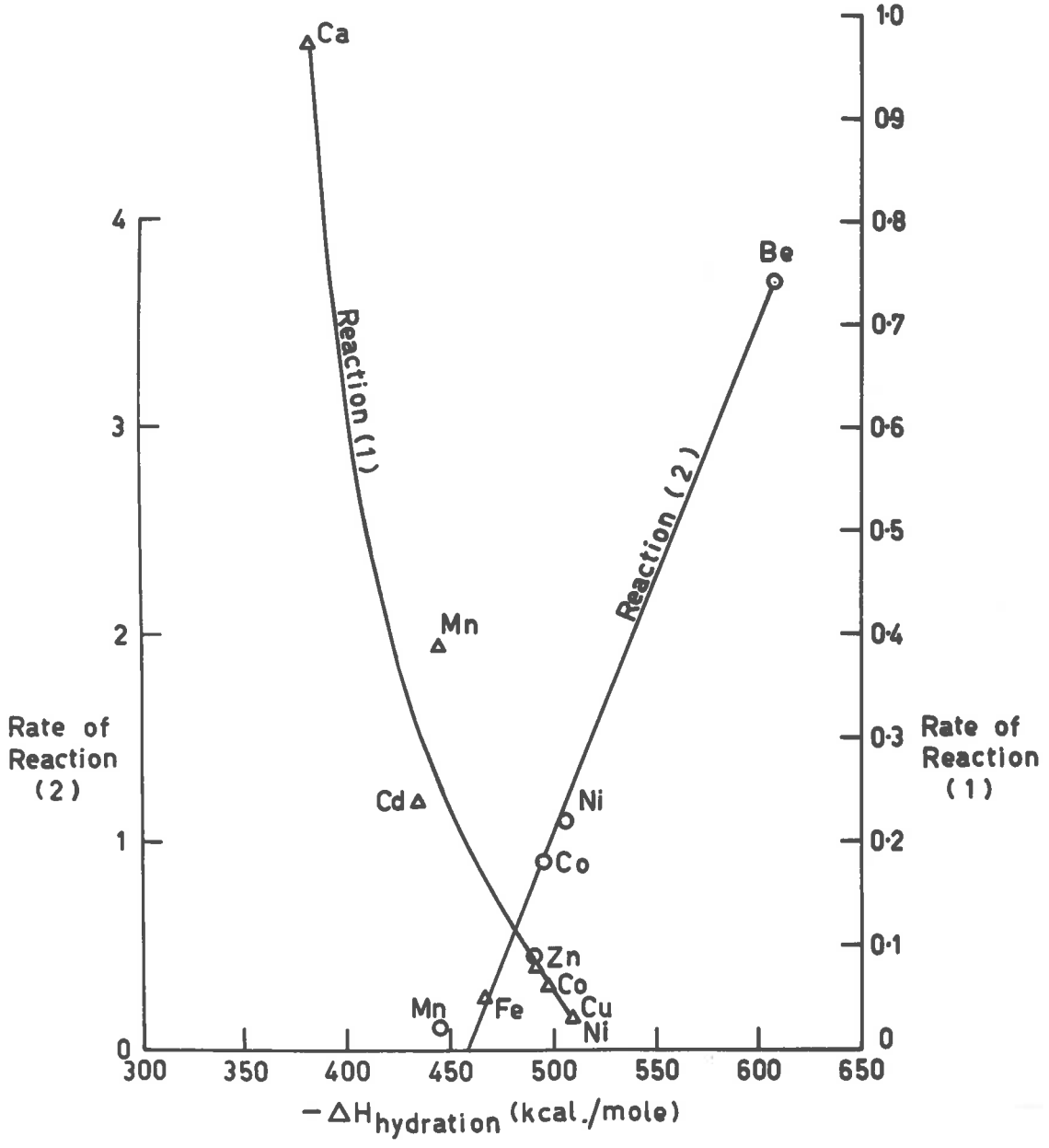

Ca
Be
Mn
Cd
Ni
Co
Zn
Co
Mn
Fe
Cu
Ni
Reaction (1)
Reaction (2)
Rate of Reaction (2)
Rate of Reaction (1)
0
1
2
3
4
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
300
350
400
450
500
550
600
650
$-\Delta H_{hydration}$ (kcal./mole)

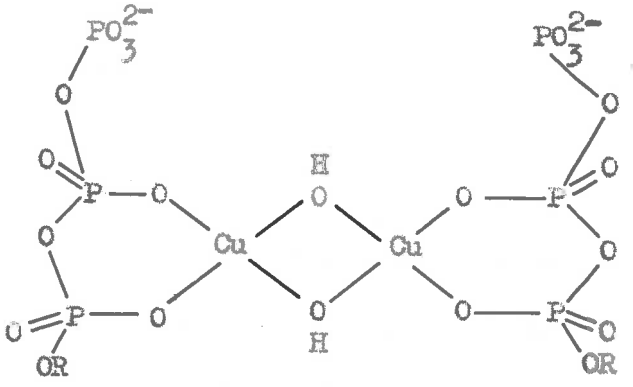
Fig. V - 5

with which an ion can be polarized. Guerdjckov (1961) has shown that the larger the heat of hydration of the ion, the smaller its polarizability becomes. If, as seems likely, the polarizability of an ion is inversely related to its polarizing effects on neighbouring ligands (see Glasstone, 1953, for a discussion on polarizability and polarizing power of ions), then higher rates of adenylyl transfer are associated wuth the presence of divalent cations with greater polarizing powers. Hammes and Kochavi (1962) have postulated a similar role for the metal ion in the hexokinase reaction, where they consider the primary role of Mg^{2+} is to polarize the O-P bond being broken, while anchoring the polyphosphate chain of ATP to the enzyme. Further work on the reactions investigated by Lowenstein (1958) and Lowenstein and Schatz (1961), where the catalysis is not complicated by the presence of an enzyme, may throw more light on the way in which metal ions aid in the transfer of phosphoryl groups from ATP.

The <u>type</u> of complex concerned in the interaction between ATP and metal ions may be important for effective adenylyl transfer to NMN A study of the nuclear magnetic resonance spectra of some of these complexes has been carried out by Cohn and Hughes (1962). They found indications that Mg^{2+}, Ca^{2+} and Zn^{2+} form complexes with the β and γ phosphate groups, that Mn^{2+} and Co^{2+} interact with α, β and γ phosphate moieties, and that of those metal ions studied, only Cu^{2+} complexes solely with the α and β phosphate residues of ATP. Since the bond between the α and β phosphorus atoms of ATP is broken during ATP:NMN adenylyl transfer, it may be advantageous to have only the β and γ groups chelated to the cation. This may be a reason for the

non-participation of Cu^{2+} in adenylyl transfer.

Recently Taqui Khan and Martell (1962) have obtained evidence that Cu^{2+} can form a polynuclear metal complex with ATP of the type



(R = adenosine residue)

Furthermore, they suggest that in the pH range 7 to 8, a large proportion of the Cu^{2+} is bound to ATP either as this dimer or as the hydroxychelates. Either of these forms may not be suitable for adenylyl transfer. It is possible that Be^{2+} behaves in a similar manner.

It has often been assumed that divalent cations can bind to the 6-amino group on the purine ring as well as to the polyphosphate chain of ATP. However, it seems to be generally accepted now that they do not interact with this amino group (see Taqui Khan and Martell, (1962); Cohn and Hughes, (1962)) except possibly in the case of Zn^{2+}. Since in general nitrogen-containing ligands have a much higher affinity for the transition elements than do oxygen ligands (Schwarzenbach, 1961), if chelation to the 6-amino group is important in the mechanism of adenylyl transfer, one would expect a change to be reflected in the

ratio of rates obtained with the various divalent cations when ITP is substituted for ATP. It was found that the ratio of rates obtained by the various cations in the ATP:NMN adenylyl transferase system is approximately the same whether the nucleotidyl donor is ATP or ITP (Tables V-1 and V-2) suggesting that the chelation of the metal ion with the 6-amino group is non-existent, as is thought to be the case, or is not important in this transfer reaction.

General Discussion

A study of the specificity of pig liver ATP:NMN adenylyl-transferase has shown that in general little lattitude is allowable in the structure of its substrates for the reaction to proceed at reasonably high rates. The points about the substrate molecules to which the enzyme has been shown to be sensitive to changes in structure are indicated in Figure VI-1, and listed below.

Nicotinamide mononucleotide

1. Carbonyl group on the substituent of $C_{(3)}$ of the pyridine ring. Substitution of the oxygen atom by sulphur lowers V_{max} and raises K_m.

2. Amino group on the substituent at $C_{(3)}$ of the pyridine ring. Substitution by a hydroxyl group raises both K_m and V_{max}. Replacement by a methyl group or a hydrogen atom raises K_m. Removal of the 3-carboxamide side chain results in complete loss of transferase activity.

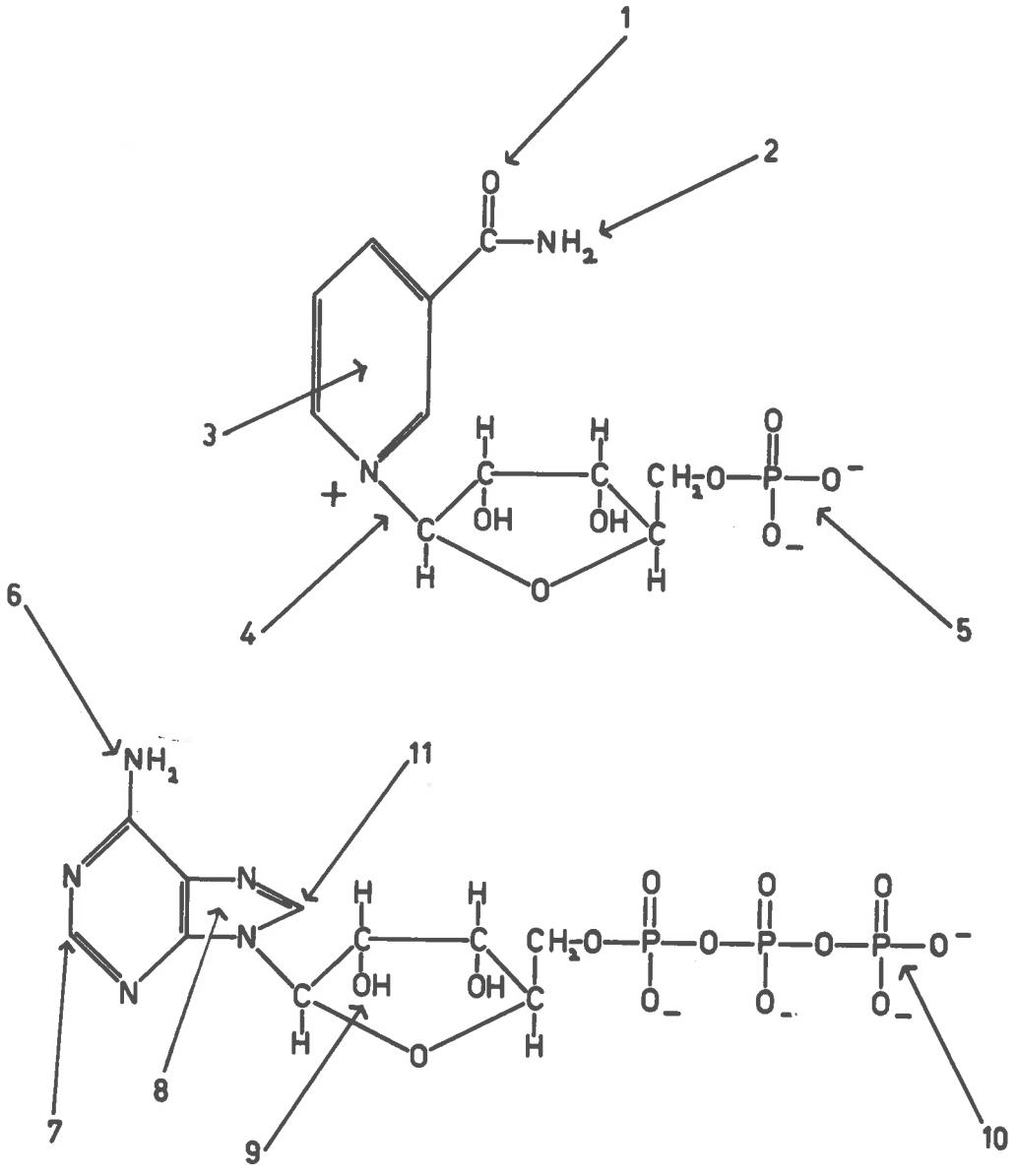
3. Reduction of the pyridinium ring lowers the rate of transfer (Kornberg, 1950).

4. Configuration about the N-ribose linkage must be β (Jackson, 1960).

5. Phosphoryl group must be present for adequate binding, as nicotinamide riboside is not an effective inhibitor. Previous studies (Atkinson, Jackson and Morton, 1961) indicated that the doubly dissociated form was the effective ionic species.

Fig. VI-1

Substrate specificity of ATP:NMN adenylyl transferase (see text).



Substrate Specificity of ATP : NMN Adenylyltransferase

Fig. VI - 1

Adenosine triphosphate

6. Amino group at the 6-position on the purine ring. Its replacement by an hydroxyl group lowers V_{max}, and, to a lesser extent, raises K_m. Substitution by a sulphydryl group lowers rate of nucleotidyl transfer.

7. Introduction of a substituent here (an amino group) effects rate of transfer only slightly.

8. The purine ring is essential. Pyrimidine analogues give no transfer reaction.

9. 2' Deoxy analogues give a lower rate of transfer.

10. γ-Phosphate group is essential for transfer. There is no transfer reaction with ADP (Kornberg, 1950).

11. $C_{(8)}$ of purine ring. Substitution of this carbon atom by nitrogen lowers the rate of transfer.

This list is not intended to be exhaustive. The studies were necessarily limited by the availability of substrate analogues. However, the number of points of sensitivity so far encountered about the substrate molecules, indicates a multipoint attachment to the active centre. It is probable that the overall shape of the substrates is important in the formation of the enzyme-substrate complex, and that the attractive forces involved are weak chamical bonds such as hydrogen bonds and electrostatic attractions, together with non-localised dispersion forces associated with "shape-matching" between the substrate and the active centre.

The indications are that certain parts of the substrates involved

are more important than others in effecting the enzymic reaction. Structural changes at 'points of sensitivity' 1, 2 and 6 have a greater effect than at 3, 7, 9 and 11 (see figure VI-1). It has been pointed out that 1 and 2 appear to require relatively polar groups for high rates of transfer, and that it is likely that dual hydrogen bonding plays a role here in attachment of the mononucleotide to the enzyme. It is known that hydrogen bonding provides stronger attractive forces than do van der Waals' forces (Bernal, 1959). If, for example, the 2'-hydroxyl group on the ribose moiety of ATP merely aids in binding by virtue of van der Waals' forces between the ATP molecule at about this point and the active centre, then one would expect its substitution by a hydrogen atom to have less effect than the substitution of the amino (or hydroxyl) group at the 3-side chain of NMN (or nicotinic acid mononucleotide) by a hydrogen atom, where hydrogen bonding is postulated

Substitution of the amino group at the 6 position of the purine ring of ATP appears to have a greater effect on V_{max} than on K_m. Thus both ITP and 6-mercapto purine riboside-5'-triphosphate are effective inhibitors of adenylyl transfer. The reason for these effects is obscure at present. It would seem that hydrogen bonding at this position is not involved, as sulphur containing groups, due to the lower electronegativity of the sulphur atom, form poorer hydrogen bonds than do nitrogen or oxygen containing residues (Boyer, 1959), yet the K_i for 6-mercapto purine-riboside-5'-triphosphate has approximately the same value as the K_m for ATP, and is lower than the K_i for ITP. However, the chemistry of a sulphydryl group positioned at the 6-position

of a purine ring is not well known as yet, and it could have special characteristics enabling firm binding somewhere at the active site. Because the hydroxyl and sulphydryl groups on the purine ring have dissociations near the physiological pH region (pK'_a 8.7 and 7.7 respectively (Fox et al., 1958)), a study of the effect of pH on inhibition and nucleotidyl transfer by both ITP and 6-mercapto purine riboside-5'-triphosphate could lead to more information on the binding of these nucleoside triphosphate analogues.

The finding that the peculiar effects of varying both Mg^{2+} and ATP concentrations (such that the total Mg^{2+} concentration is always less than the total ATP concentration) on the rate of adenylyl transfer can be interpreted in terms of the Mg-ATP complex being the true substrate and uncomplexed ATP acting as a competitive inhibitor, serves as a pointer to the role of the metal ion in the transfer reaction. Since the suggested K_i for ATP (2mM) is not so very different from the K_m of MgATP (0.12mM), then it would seem that ATP can be bound to the active centre in the absence of metal ion and that this metal ion does not play a substantially greater part in binding than any of the 'points of sensitivity' listed above. Yet a metal ion such as Ni^{2+}, Co^{2+}, Zn^{2+} Mg^{2+}, Mn^{2+} or Cd^{2+} is absolutely essential for the reaction.

The fact that substitution of Mg^{2+} by Ni^{2+} or Co^{2+} does not effect the Km for ATP, but greatly increases V_{max}, suggests that the metal ion plays an important part in the breakdown of the enzyme-substrate complex. For the reaction catalysed by ATP:NMN adenylyltransferase, the studies of metal ion specificity reported here indicate a relationship between V_{max} and the heat of hydration of the metal ion used. Because the heat

of hydration for a cation may be an expression if its polarizing power, it is suggested that the function of the metal ion is to polarize certain portions of the polyphosphate chain of ATP during nucleophilic attack by NMN and thus aid in the bond breaking step (cf. Hammes and Kochavi, 1962). The greater the polarizing power of the metal ion, the easier nucleophilic attack becomes, and the higher the rate of transfer. A kinetic study of the relationships between NMN and metal ions, similar to that carried out for ATP above, may provide a fuller understanding of the mechanism of adenylyl transfer. It seems that the 5'-phosphoryl group is essential for adequate binding of NMN. An investigation of the effect of substituting Mg^{2+} by Ni^{2+} or Co^{2+} on the K_m for NMN could indicate whether or not the metal ion plays a part in this binding. The V_{max} of course will be increased.

The role the enzyme molecule plays in effecting the actual nucleotidyl transfer step is unknown as yet. Presumably, besides forming a complex with ATP and NMN, such that the two substrates are in an advantageous spatial relationship to each other for a transfer reaction, the groups at the active site also effect the positioning of the Mg^{2+} ion along the polyphosphate chain of ATP to give the polarization of bonds needed for nucleophilic attack at the α-phosphorus atom of ATP.

In analysing the specificity relationships for the pig liver enzyme it must be remembered that starch gel electrophoresis of some preparations indicated the presence of two or three isoenzymes. It has been shown in at least one case that the members of an isoenzymic series, separated by means of starch gel electrophoresis, may exhibit

different substrate specificities (Allen, 1961). It is clear then that caution is needed in drawing conclusions from the present specificity studies and that this point requires further investigation. It would be of interest to test the apparent isoenzymes of ATP:NMN adenylyltransferase for their relative activities with ATP and deoxy ATP, and with NMN and nicotinic acid mononucleotide.

The results presented here suggest that nicotinic acid mononucleotide has both a higher K_m and higher maximal velocity than NMN in the reaction catalysed by the pig liver transferase. The higher V_{max} has been attributed to the greater overall nucleophilic character of the nicotinic acid analogue, and the higher K_m (if this involves a lower affinity) to the diminished ability of the $C_{(3)}$ substituent of nicotinic acid mononucleotide (compared to NMN) to form hydrogen bonds at a physiological pH. That the same enzyme is responsible for adenylyl transfer to NMN and to nicotinic acid mononucleotide (Preiss and Handler, 1958) is further supported by the finding that both the K_i and K_m for the nicotinic acid analogue are approximately 3.5 fold greater than the K_m for NMN.

The specificity of the nuclear ATP:NMN adenylyltransferase is such, that there appears to be no reason to suppose that the enzyme, in vivo, would use one mononucleotide much more effectively than the other. The only factor which will determine this is the availability of NMN and nicotinic acid mononucleotide in the animal tissue. An enzyme catalysing the synthesis of nicotinic acid mononucleotide is known (Preiss and Handler, 1958). However no metabolic pathway capable of synthesizing NMN under physiological conditions has yet

been described (Imsande and Handler, 1961). If the synthesis of NAD proceeds only via the nicotinic acid analogues, as suggested by Preiss and Handler (1958), then it is necessary to consider another function of the transferase in postulating an evolutionary advantage in a 'physiological' K_m for NAD (and NMN), since NAD has been found present in all tissues which have been tested. This function could be to provide ATP, from NAD, for synthetic reactions taking place within the nucleus at certain times in the life of a cell.

It has been suggested that an effective inhibitor of ATP:NMN adenylyltransferase could find applications in chemotherapy (Morton, 1958). 6-Mercaptopurine riboside-5'-triphosphate appears to be a potent inhibitor of the enzyme (Atkinson et al., 1961 a; Atkinson et al., 1962), a finding which could explain some of the effects of 6-mercaptopurine when administered to rapidly growing cells. The increase in liver NAD following injection of mice with nicotinamide was found by Kaplan et al., (1956) to be reduced by 6-mercaptopurine, which also tended to reduce the subsequent dimunition of the high NAD level. However, the conclusions to be drawn from these observations is complicated by the fact that 6-mercaptopurine analogues can also inhibit certain steps in the synthesis of AMP (Hampton, 1962). To provide a specific inhibitor for the transferase it would be preferable to find an analogue of NMN with a sufficiently high affinity for the enzyme. All the analogues of NMN examined so far have a relatively low affinity for the transferase and do not fulfill the requirements for a specific and effective inhibitor.

Summary

1. Methods for the purification of ATP:NMN adenylyltransferase from pig liver nuclei are described.

2. A histochemical technique for the demonstration of areas of transferase activity on starch gel has been developed. Starch gel electrophoresis of the enzyme preparations show that they are not homogeneous.

3. Nicotinamide guanine dinucleotide and the mononucleotides of thionicotinamide, 3-acetylpyridine and 3-aldehydepyridine have been prepared enzymically and characterised.

4. A study of the specificity of the transferase has shown that many nicotinamide mononucleotide and adenosine triphosphate analogues will participate in the transfer reaction. A kinetic investigation into the ability of some of these analogues to act as substrate or inhibitor has indicated the relative importance of some of the structural units of the natural substrates in aiding binding to the enzyme.

5. The metal ion requirements for the transfer reaction can be satisfied by Ni^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+} or Mn^{2+}. The kinetics of the rate of transfer in the presence of these cations is consistent with the possibility that the cation-ATP complex is the true form of the substrate.

6. The metal ion does not appear to play a more important role in the binding of nucleoside triphosphate to the active centre

than any of the other structural units of the cation-ATP complex. It is thought to be involved in the polarization of bonds about the α-phosphorus atom of ATP that may be necessary for the nucleophilic attack by NMN to result in adenylyl transfer.

7. The significance of these findings is discussed.

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