ANIMAL POLYNUCLEOTIDE PHOSPHORYLASE

Thesis presented by

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to the

Division of Sciences
School of Graduate Studies

in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

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ACKNOWLEDGEMENTS

I wish to thank Dr. Peter S. Fitt for his patient and enthusiastic guidance of the work presented here.

My thanks to all the professors in the Department for the use of their instruments.

I acknowledge with thanks the receipt of a Province of Ontario Graduate Student Fellowship.

SUMMARY

1) Polynucleotide phosphorylase has been shown to be present unequivocally in rat liver mitochondria.

2) It is localized exclusively in the inner membrane of the organelle.

3) The inner membrane enzyme has been solubilized with Triton X-100 and partially purified and has the following properties:

(i) It was obtained in a particulate form containing little or no lipids.

(ii) It catalyses phosphorolysis of poly A, poly C, poly U and high molecular weight RNA but not poly G or DNA.

(iii) It requires Mg^{2+} for activity and the latter could be replaced by Mn^{2+} to some extent, but not by Ca^{2+}.

(iv) The purified polynucleotide phosphorylase has pH optimum of 8.2 while the membrane-associated enzyme has an optimum at pH 7.8. This discrepancy may be partly due to the presence of phosphatases, in the membrane, which degrade nucleotides and have pH optimum at 8.0. The phosphatases were completely removed during purification.

(v) It does not catalyse nucleoside diphosphate polymerization.

(vi) The particulate enzyme is associated with ADP/P_i and ATP/P_i exchange activities but these exchanges may be due to separate enzymes.

(vii) The particulate enzyme is contaminated by adenylate kinase and an endonuclease that degrade poly A to oligonucleotide

ABBREVIATIONS

RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
AMP, GMP, IMP, UMP, CMP	The 5'-phosphates of adenosine, guanosine, inosine, uridine, cytidine.
ADP, etc.	The 5'-diphosphate of adenosine, etc.
dADP, etc.	The 5'-diphosphate of deoxyadenosine, etc.
ATP, etc.	The 5'-triphosphates of adenosine, etc.
NDP	Nucleoside diphosphates
NTP	Nucleoside triphosphates
ApA	Adenylyl-(3'-5'-)adenosine
ApApdA	Adenylyl-(3'-5')adenylyl-(3'-5'-)deoxyadenosine
t-RNA	Transfer RNA
m-RNA	Messenger RNA
Poly A, etc.	Linear 3'-5' polymer of adenylic acid, etc.
Poly (A,C), etc.	Linear 3'-5' copolymer of adenylic acid and cytidylic acid, etc., in random sequence.
P_i	Inorganic orthophosphate
$^{32}P\text{-}P_i$	^{32}P-labelled inorganic orthophosphate
NADH	Nicotinamide-adenine dinucleotide, reduced.
DEAE-cellulose	Diethylaminoethyl-cellulose
TLC	Thin layer chromatography
Tris-	Tris(Hydroxymethyl)aminomethane

4) Mitochondrial polynucleotide phosphorylase has been shown to be widely distributed in various animal species and in different rat tissues.

5) Membrane bound polynucleotide phosphorylase has been isolated from crude nuclear preparation of rat liver and guinea-pig liver. These enzymes may be from mitochondrial contaminations, but conclusive proof of such an origin could not be obtained and the possibility exists that polynucleotide phosphorylase may also occur in the nuclei of animal cells.

6) Polynucleotide phosphorylase activity could not be detected in glycogen particles, lysosomes, microsomes, peroxisomes or soluble fraction.

7) The Fiske and SubbaRow and Lowry and Lopez procedures have been modified for the determination of inorganic phosphate in the presence of Triton X-100.

ABBREVIATIONS

RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
AMP,GMP,IMP,UMP,CMP	The 5'-phosphates of adenosine, guanosine, inosine, uridine, cytidine.
ADP, etc.	The 5'-diphosphate of adenosine, etc.
dADP, etc.	The 5'-diphosphate of deoxyadenosine, etc.
ATP, etc.	The 5'-triphosphates of adenosine, etc.
NDP	Nucleoside diphosphates
NTP	Nucleoside triphosphates
ApA	Adenylyl-(3'-5'-)adenosine
ApApdA	Adenylyl-(3'-5')adenylyl-(3'-5'-)deoxyadenosine
t-RNA	Transfer RNA
m-RNA	Messenger RNA
Poly A, etc.	Linear 3'-5' polymer of adenylic acid, etc.
Poly (A,C), etc.	Linear 3'-5' copolymer of adenylic acid and cytidylic acid, etc., in random sequence.
P_i	Inorganic orthophosphate
$^{32}P\text{-}P_i$	^{32}P-labelled inorganic orthophosphate
NADH	Nicotinamide-adenine dinucleotide, reduced.
DEAE-cellulose	Diethylaminoethyl-cellulose
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I. INTRODUCTION

A. Bacterial polynucleotide phosphorylase

Polynucleotide phosphorylase (nucleoside diphosphate-polynucleotide nucleotidyltransferase EC 2.7.7.8) was first discovered by Grunberg-Manago and Ochoa in 1955. They found that dialysed crude extracts of Azotobacter vinelandii catalysed exchange of ^{32}P-labelled inorganic orthophosphate with the terminal phosphate of ADP, UDP, IDP and CDP. This exchange was specific for the nucleoside diphosphates: no similar reaction occurred when the nucleoside diphosphates were replaced by the triphosphates or monophosphates. When they studied the properties of a partially purified enzyme preparation, they found that it catalysed the formation of high molecular weight polyribonucleotides from ribonucleoside 5'-diphosphates in the presence of Mg^{2+}, with the simultaneous release of inorganic orthophosphate. The enzyme also catalysed a phosphorolytic reaction, releasing nucleoside 5'-diphosphates from polyribonucleotides, in the presence of orthophosphate and Mg^{2+}. The overall reaction is represented by the equation

$$n\text{NDP} \rightleftharpoons^{Mg^{2+}} (\text{NMP})n + n\text{Pi}$$

Since this reversible reaction was similar to that of the polysaccharide phosphorylase reaction, they named the enzyme polynucleotide phosphorylase (Grunberg-Manago and Ochoa, 1955; Grunberg-Manago, Ortiz and Ochoa, 1956). Soon after the publication of Grunberg-Manago and Ochoa's paper, papers dealing with polynucleotide phosphorylases from other bacteria appeared in the literature; Escherichia coli (Littauer, 1956; Littauer and

Kornberg, 1957); Micrococcus lysodeikticus (Beers, 1956); Clostridium perfringens (Dolin, Godinicaux and Grunberg-Manago, 1961); and it was found to be widely distributed among aerobic and anaerobic bacteria (Grunberg-Manago, 1963).

There have been two reports in the literature, to date, that polynucleotide phosphorylase was undetectable in certain strains of bacteria. However, the enzyme was found in both cases, when more careful studies were undertaken. Ochoa et al. (1961) could not detect polynucleotide phosphorylase in Lactobacillus arabinosus. Recently, Thang, Dondon and Grunberg-Manago (1969) showed that polynucleotide phosphorylase was present in this bacterium. The enzymic activity was low (about one percent of that found in A. vinelandii) and it had somewhat different properties as compared with other bacterial polynucleotide phosphorylases that had been studied. It catalysed phosphorolysis of oligonucleotides and not high molecular weight polynucleotides. It had also been reported that polynucleotide phosphorylase was absent in E. coli Q13, an E. coli mutant lacking ribonuclease I (Ben-Hamid and Schlessinger, 1966). The inability to detect polynucleotide phosphorylase in crude extracts of this E. coli mutant was shown to be due to the presence of very high nucleoside diphosphate pyrophosphatase activities which were higher than that found in the wild strain E. coli (Natori and Mizuno, 1967). The presence of polynucleotide phosphorylase in E. coli Q13 had been confirmed by partial purification of the enzyme (Hsieh and Buchanan, 1967; Thang, Thang and Grunberg-Manago, 1967; Reiner, 1969(a)).

Polynucleotide phosphorylases from other E. coli mutants have also been isolated and studied (Reiner, 1969, a,b; Castles and Singer, 1968). Recently, polynucleotide phosphorylase was isolated and purified from the extreme halophile Halobacterium cutirubrum (Peterkin and Fitt, 1971) and, from the blue-green alga Anacystis nidulans (Capesius and Richter, 1967). The blue-green alga polynucleotide phosphorylase had properties similar to those of the normal bacterial enzyme. Hence, polynucleotide phosphorylase has been found in all the procaryotic cells that have been studied and Thang, Dondon and Grunberg-Manago (1969) have suggested that it is present in all bacteria.

Bacterial polynucleotide phosphorylase catalyses the following reactions:

$$nNDP \xrightleftharpoons{Mg^{2+}} (NMP)_n + nP_i \qquad (1)$$

$$NMP\text{-}P + {}^{*}P_i \xrightleftharpoons{Mg^{2+}} NMP\text{-}{}^{*}P + P_i \qquad (2)$$

The overall reaction catalysed by these enzymes is shown by equation (1); where NDP represents ribonucleoside 5'-diphosphate; NMP, ribonucleoside 5'-monophosphate units in polyribonucleotides, and P_i, inorganic orthophosphate. The forward reaction, the polymerization reaction, gives rise to a homopolymer when a single nucleoside diphosphate species is used as substrate and to co-polymers if two or more different species of nucleoside diphosphate are used. The reverse of the reaction, the phosphorolysis of polyribonucleotide in the presence of inorganic orthophosphate, gives nucleoside 5'-diphosphate as the product. An exchange reaction (equation 2) whereby the terminal phosphate of ribonucleoside 5'-diphosphate exchanges with inorganic ortho-

phosphate is also catalysed by polynucleotide phosphorylase. This reaction is specific for nucleoside diphosphate and can be observed when ^{32}P-labelled inorganic orthophosphate is used for study. A fourth reaction, transnucleotidation, had been reported with the A. vinelandii enzyme (Singer et al., 1959). The latter catalysed the transfer of nucleoside monophosphate units from a polynucleotide donor to a polynucleotide acceptor without the intervention of inorganic orthophosphate or nucleoside diphosphate as shown in the following equation, with a trinucleotide substrate as an example:

$$pApApA + pApApA \rightleftharpoons^{Mg^{2+}} pApApApA + pApA$$

In all the reactions catalysed by polynucleotide phosphorylase the presence of divalent cation is required, usually magnesium ions (Grunberg-Manago, 1963; Babinet et al., 1965).

In the following sections, I will attempt to give a brief account of the various reactions catalysed by bacterial polynucleotide phosphorylase. Enzymes from four different bacteria have been purified and studied extensively. The properties of the enzymes from E. coli, A. vinelandii and M. lysodeikticus are fairly similar, although there are some differences in the detailed kinetics of their action. The enzyme purified from Cl. perfringens differs considerably from the polynucleotide phosphorylases of other bacteria (Grunberg-Manago, 1963; Fitt, Dietz and Grunberg-Manago, 1968).

1) The polymerization reaction

$$nNDP \xrightarrow{Mg^{2+}} (NMP)_n + nPi$$

Detailed studies have shown that polynucleotide phosphorylase polymerizes ribonucleoside 5'-diphosphates to give high molecular weight polyribonucleotides (Grunberg-Manago, 1963). This reaction is specific for ribonucleoside diphosphates and ribonucleoside tri- or monophosphates are not substrates. The polyribonucleotides obtained are linear polymers, with molecular weight in the range 30,000 - 2,000,000, and are composed of ribonucleotides linked by 3'-5'-phosphodiester bonds similar to those of natural RNA.

The enzyme polymerizes ADP, UDP, IDP or CDP readily to produce the homopolymers poly A, poly U, poly I and poly C, respectively. Polymerization of GDP is difficult or does not occur under the same conditions used for the other nucleoside diphosphates, and will be discussed in more detail later.

Copolymers were obtained when a mixture of two or more nucleoside diphosphates was used as substrate. It was shown by Ortiz and Ochoa (1959) that, when a mixture of all four common nucleoside diphosphates (ADP, GDP, UDP and CDP) was used as substrate, the incorporation of the nucleotides occurred randomly. The base composition of the copolymer produced, poly (A,G,U,C), depended on the relative concentrations of the various nucleoside diphosphates used only, and was unaffected by the addition of RNA to the reaction mixture (Ochoa and Mii, 1961). The synthesis of the copolymer poly (A,G,U,C) was considerably slower than the rate of formation of homopolymers. To obtain a similar rate of synthesis, twenty to thirty times more enzyme had to be used for the synthesis of poly (A,G,U,C) from equimolar concentrations of ADP, GDP, UDP, and CDP. This is believed (Grunberg-Manago,

1963) to be due to the presence of GDP. GDP is polymerized with difficulty under the same conditions used for the polymerization of the other nucleoside diphosphates and its presence hindered the synthesis of copolymer (Grunberg-Manago, Ortiz and Ochoa, 1956; Ochoa and Mii, 1961). With saturating concentrations of the nucleotides, the rates of formation of poly A, poly U, poly C and poly I were about the same and, the rate of polymerization of the copolymer, poly (A, U), was about half of the rate for the synthesis of homopolymers (Grunberg-Manago, 1961).

a) The effects of primers on the polymerization reaction

Earlier work showed that partially purified polynucleotide phosphorylase obtained from A. vinelandii (Ochoa and Mii, 1961), M. lysodeikticus (Singer and Guss, 1962, Singer and O'Brien, 1963) and E. coli (Grunberg-Manago, 1963) catalysed the polymerization reaction only after an initial lag period. This lag phase could be overcome by the addition of oligonucleotides or long chain polyribonucleotides to the reaction mixture (Singer, Heppel and Hilmoe, 1957; Mii and Ochoa, 1957). The presence of the lag phase was noted when ADP, UDP, CDP or IDP were used as substrates. With GDP as substrate, little or no polymerization reaction was observed even when a crude extract was used. However, limited but definite polymerization of GDP occurred when an oligonucleotide primer was added to the reaction mixture (Singer, Hilmoe and Grunberg-Manago, 1960). Further study of the mechanism of stimulation by oligonucleotides in the presence of the A. vinelandii enzyme (Singer, Heppel and Hilmoe, 1957, 1960) showed that oligonucleotides (pApA, pApApA, etc.) with free 3'-terminal hydroxyl groups were incorporated into the growing polymer

chain. Hence, these oligonucleotides act as primers for the polymer formation. Oligonucleotides with free hydroxyl group at the 5'-terminus of the chain as well as free hydroxyl group at the 3'-terminus (ApA, ApApA, etc.) could also act as primers. Oligonucleotides with 3'-terminal phosphate groups (ApAp, ApApAp, etc.) could also overcome the lag phase observed in the polymerization reaction by a partially purified A. vinelandii enzyme, but they were not incorporated into the polymer formed. The polymerization of GDP was stimulated by oligonucleotides with free hydroxyl group at the 3'-terminus but not by oligonucleotides with 3'-terminal phosphate groups.

The mechanism whereby oligonucleotides with 3'-phosphate termini stimulated the polymerization reaction of A. vinelandii polynucleotide phosphorylase is not known. These oligonucleotides acted as inhibitors for the polymerization of nucleoside diphosphates by partially purified M. lysodeikticus polynucleotide phosphorylase (Beers, 1961; Singer and O'Brien, 1962). The polymerization reaction catalysed by the M. lysodeikticus enzyme was stimulated by oligonucleotides with free hydroxyl group at the 3'-terminus.

Crude preparations of bacterial polynucleotide phosphorylases polymerized nucleoside diphosphates readily. When the enzymes were partially purified, they acquired a lag phase which could be relieved by the addition of oligonucleotides with free terminal 3'-hydroxyl group. These oligonucleotides were found to be incorporated into the polymer synthesized, even when they were added to a crude enzyme preparation. These observations suggested

that polynucleotide phosphorylase catalysed only the elongation of preformed polynucleotide chains and might not be able to catalyse de novo synthesis (Grunberg-Manago, 1962). The partially purified enzyme which is stimulated by oligonucleotides in the polymerization reaction is referred to as 'primer-dependent enzyme'.

Recently, primer-independent polynucleotide phosphorylase has been isolated and purified from M. lysodeikticus (Klee, 1967; Fitt and Fitt, 1967; Moses and Singer, 1970) and A. vinelandii (Gajda and Fitt, 1969; Gajda, Zaror de Behrens and Fitt, 1970). Olmsted and Lowe (1959) demonstrated that trypsin digestion caused considerable change in the substrate specificity of M. lysokeikticus polynucleotide phosphorylase. The more recent studies (Fitt and Fitt, 1967; Fitt, Fitt and Wille, 1968) demonstrated that the effects observed by Olmsted and Lowe were due to change in the primer requirement of the enzyme.

It was shown with the M. lysodeikticus enzyme that the primer-independent enzyme could be converted to the primer-dependent form by proteolysis with trypsin, prolonged ultracentrifugation or treatment with 1 M guanidine hydrochloride (Klee, 1967). The primer-dependent form obtained after tryptic digestion could be reconverted back to the primer-independent form by treatment with thiol reagents (Klee and Singer, 1968). The primer-dependent and primer-independent enzymes could be separated by disc gel electrophoresis. The primer-dependent enzyme had the same electrophoretic mobility as the primer-independent form after treatment with thiol reagents. Fitt and Fitt (1967) and Fitt, Fitt and Wille (1968) also studied

the effect of proteolysis on M. lysodeikticus polynucleotide phosphorylase. They found that controlled, limited trypsin digestion caused a marked increase in the primer requirement for CDP polymerization but had no effect on the ADP polymerization. This enzyme migrated as a single band with the same mobility as that of the untreated enzyme, in disc gel electrophoresis. On prolonged digestion, the primer requirement for ADP polymerization increased and a band of activity with increased electrophoretic mobility was observed. This is in agreement with the independent observations of Klee (1967). α-Chymotrypsin digestion also caused increase in primer requirement for both ADP and CDP polymerization but, unlike trypsin digestion, several enzymically active bands with greater mobilities were seen on polyacrylamide gel disc electrophoresis.

The A. vinelandii polynucleotide phosphorylase also became primer-dependent after trypsin digestion or on prolonged storage (ageing), at 4°C, of the highly purified enzyme (Gajda and Fitt, 1969; Gajda, Zaror de Behrens and Fitt, 1970). The development of primer requirements were accompanied by marked change in the electrophoretic mobilities of the enzyme in disc gel electrophoresis. However, the primer-dependent enzyme could not be reconverted back to the primer independent form by thiol reagents.

It was also reported that E. coli polynucleotide phosphorylase became more primer-dependent after digestion with trypsin and with E. coli B protease I (Thang, Dondon and Godefroy-Colburn, 1971). On extensive digestion, the enzyme became completely primer-dependent with no activity in the absence of oli-

gonucleotide primer. The digested enzyme showed an increase in electrophoretic mobility. Thiol reagents could not reconvert the digested enzyme to the untreated form. Similar change in properties of the native, purified, enzyme with time was observed when it was stored at 4°C. These changes in properties were very similar to that observed for the A. vinelandii enzyme discussed above. These observations led the authors (Thang, Dondon and Godefroy-Colburn, 1971) to suggest that the increase in primer-dependency and the increase in electrophoretic mobility of aged enzymes were due to contaminating protease attached to the purified enzyme preparation. However, no attempt was made to show that protease was definitely present in their enzyme preparations.

From the discussion given above on the effect of proteolytic enzymes on primer dependency of polynucleotide phosphorylase, one may conclude that the primer-dependent enzymes obtained were due to proteolytic enzyme digestion of polynucleotide phosphorylase during purification.

Present evidence suggests that polynucleotide phosphorylase, whether primer-dependent or primer-independent, may catalyse the de novo synthesis of polyribonucleotides. The de novo synthesis catalysed by, either the primer-dependent or primer-independent, M. lysodeikticus enzyme produced high molecular weight polynucleotides. No short chain intermediates were detected at any time during the reaction. This type of synthesis where mononucleotide units were added progressively to the end of a growing polynucleotide chain until high molecular weight polymers were formed was

termed processive (Moses and Singer, 1970). In the presence of oligonucleotide primer, the primer-dependent enzyme catalysed the addition of mononucleotide units to the oligonucleotide primer and short chain polynucleotides were formed and the reaction is non-processive. Low molecular weights oligonucleotides of various chain lengths could be synthesized by increasing the ionic strength of the reaction mixture and oligonucleotides of different chain length could be synthesized by varying the ionic strength (Thach and Doty, 1965; Thach, 1966). In the presence of oligonucleotides, the primer-independent enzyme did not incorporate the oligonucleotides into the polynucleotide chain. Polymer synthesis in this case was processive. This processive synthesis of polynucleotides was also found with E. coli B polynucleotide phosphorylase (Thang, Harvey and Grunberg-Manago, 1970).

It is relatively easy to synthesize high molecular weight homopolymers with ADP, UDP, CDP or IDP as substrates. The polymerization of GDP was rather difficult (Grunberg-Manago, 1963) but the reaction was stimulated by primer. Even in the presence of primer, only short chain oligonucleotides could be synthesized under normal conditions for polymerization. In order to explain this unusual reaction with GDP as substrate, it was suggested that the poly G synthesized, which had been shown to have a high degree of secondary structure, inhibited the enzyme reaction (Fresco and Massoulié, 1963; Grunberg-Manago, 1963). Fresco and Su (1962) reported that polymerization of GDP could occur in the absence of primer if the concentration of GDP and Mg^{2+} were low

and in the presence of large amounts of enzyme. The products produced were of low molecular weight. The ability to synthesize high molecular weight poly G was reported by Thang, Graffe and Grunberg-Manago (1965) using E. coli polynucleotide phosphorylase. The enzymic reaction was carried out at high temperature (60°C) and Mn^{2+} replaced Mg^{2+}. At this temperature, the rate of reaction with Mn^{2+} was six times greater than with Mg^{2+}, although at 37°C replacement of Mg^{2+} with Mn^{2+} did not alter the rate of reaction appreciably. The high molecular weight poly G synthesized had sedimentation coefficient of about 8S (Thang, Graffe and Grunberg-Manago, 1965) and the average chain length was greater than 100 (Pochon and Michelson, 1965).

b) The effect of polybases on the polymerization reaction

Clostridium perfringens polynucleotide phosphorylase differed from the other bacterial enzymes that had been studied (Dolin, Godinieaux and Grunberg-Manago, 1961; Dolin, 1961 and 1962). The polymerization of ADP or GDP was markedly stimulated by polybases such as polylysine, polyornithine and polyvinylamine, whereas UDP and CDP polymerization was completely inhibited. This effect was studied in detail by Fitt, Dietz and Grunberg-Manago (1968). They found that both polylysine and inorganic salts increased the rate of polymerization of ADP. In the absence of any activator, the polymerization reaction was preceded by a very short lag phase followed by a rapid transition to a linear rate. Poly A could remove this lag phase but it did not stimulate the reaction appreciably. In the presence of sodium

chloride (0.2M), the rate of reaction was increased but a considerable lag phase was initially observed. The reaction in the presence of polylysine was twice as fast as that in the presence of sodium chloride and there was no lag phase. Both polylysine and salt lowered the Km but only the former increased the V_{max}. The ability of polylysine to stimulate the polymerization of ADP increased with increasing chain length of the polybases up to a maximum chain length of 20-30 lysyl residues (mol. wt. 3000); all the polymers with molecular weights 3000 or above were equally active (Fitt and Wille, 1969b). Fitt and Wille (1969a,b) found that, on trypsin digestion, a rapid loss of the polylysine-stimulated activity occurred while the activity without the activator was affected more slowly. The purified enzyme did not separate into two components during sucrose density gradient centrifugation or Sephadex G-200 gel filtration. It was concluded that charge effects on the enzyme itself rather than the existence of two forms of the enzyme were responsible for the stimulation by polybases and salts. In the case of A. vinelandii and E. coli polynucleotide phosphorylase, there appeared to be slight activation by polylysine (Grunberg-Manago, 1963). There was no such effect of polylysine on the M. lysodeikticus enzyme (Singer and O'Brien, 1963).

2) The phosphorolysis reaction

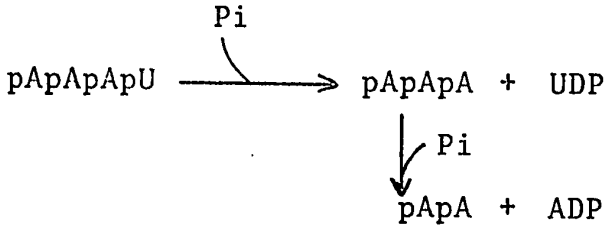
$$(NMP)_n + nPi \xrightarrow{Mg^{2+}} nNDP$$

Polynucleotide phosphorylase catalyses the phosphorolytic degradation of polyribonucleotide to produce nucleoside 5'-diphos-

phate as the main product of the reaction. The reaction requires a divalent cation, usually Mg^{2+} but other divalent cations, Mn^{2+}, Co^{2+}, Cd^{2+} and Zn^{2+}, can replace Mg^{2+} to some extent. Mn^{2+} is the most suitable divalent cation to replace Mg^{2+}. There was no reaction with Ca^{2+} (Babinet et al, 1965). Phosphorolysis was specific for polyribonucleotides and DNA was not phosphorolysed (Grunberg-Manago, 1963). A detailed study of the phosphorolysis of various types of polynucleotides was made by Grunberg-Manago (1959). She found that the single stranded homopolymers poly A, poly U, poly C and poly I were phosphorolysed readily. Copolymers like poly (A,U) and poly (A,U,G,C) were phosphorolysed more slowly. RNAs isolated from natural sources were phosphorolysed even slower than the copolymer. The slow rate of phosphorolysis of natural RNA was due to the presence of secondary structure. Poly A, poly U, poly C and poly I had little secondary structure in the conditions used for phosphorolysis. The effect of the secondary structure of polynucleotides on phosphorolysis was also studied. When the double stranded poly A: poly U was used as substrate, it was phosphorolysed at a rate one-third that for the phosphorolysis of poly A or poly U. Poly I was phosphorolysed readily at low ionic strength but when the potassium chloride concentration was increased to 0.6M, the rate of reaction was very slow. At this high ionic strength, poly I has a triple stranded helical structure (Rich, 1958). At the same salt concentration, the phosphorolysis of poly A and poly U were slightly stimulated. The structure of poly A and poly U were not affected greatly at this salt concentration (Grunberg-Manago, 1959, 1963). Transfer

RNA (t-RNA) which had considerable secondary structure was phosphorolysed slowly and, even after long incubation, it was only 40% degraded at 37°C. When the temperature of the reaction was raised to 45°C, t-RNA was totally phosphorolysed (Thang et al, 1967) and there was a further increase in the rate of phosphorolysis as the temperature was raised from 45° to 60°C. At 37°C the rates of phosphorolysis of natural RNAs were in the order: tobacco mosaic virus RNA > microsomal RNA > t-RNA (Grunberg-Manago, 1959) and there was a definite relationship between the rate of phosphorolysis, at different temperatures, and the secondary structures of the RNAs (Michelson, 1963). Poly G, with extensive secondary structure (Michelson, 1963; Yang and Samejima, 1969) was phosphorolysed very slowly (Grunberg-Manago, 1959).

The mechanism for the phosphorolysis of high molecular weight homopolymers and oligonucleotides has been studied in detail. Early studies on the phosphorolysis of well defined oligonucleotides, of the type, pApApA, pApApApU, etc., had shown that phosphorolysis started from the 3'-end. The cleavage of the oligonucleotides occurred randomly as shown below.



On prolonged incubation, all the oligonucleotides were reduced to the nucleoside diphosphates and the dinucleotide pApA. This dinucleotide was resistant to phosphorolysis. Oligonucleotides with phosphate esterified at the 3'-end were not phosphorolysed

and they inhibited the phosphorolysis of oligonucleotides and high molecular weight polymer. The presence of a 5'-terminal phosphate group had no effect on phosphorolysis of the oligonucleotides (Hilmoe, 1959; Singer, Hilmoe and Grunberg-Manago, 1960; Grunberg-Manago, 1963). More detailed study of the kinetics of phosphorolysis has shown that oligonucleotides with phosphate esterified at the 3'-end are competitive inhibitors with respect to oligonucleotides substrates and non-competitive inhibitors with respect to inorganic phosphate (Chou and Singer, 1970a,b). The stepwise phosphorolysis of oligonucleotides with chain length up to six proceeded by a random mechanism as described above.

The mechanism for the phosphorolysis of high molecular weight polyribonucleotide was different from that for oligonucleotides. Thang et al. (1967) have shown that the phosphorolysis of t-RNA at high temperature (45° to 60°C) by E. coli polynucleotide phosphorylase caused complete degradation of the t-RNA molecule. They also found that the rate of phosphorolysis and the rate of release of ^{32}pA from t-RNA-pCpC^{32}pA were very similar. This indicated that polynucleotide phosphorylase degrades the whole t-RNA molecule before it attacks another t-RNA molecule. This concept was further strengthened by the parallel loss of amino acid acceptor activity of the t-RNA with the time of phosphorolysis of the t-RNA. This mode of degradation was termed processive degradation by Klee and Singer, 1968. These authors confirmed the observation of Thang et al. (1967) with M. lysodeikticus polynucleotide phosphorylase. They found that the degradation of poly A was processive and no oligonucleotides, other than the

resistant dinucleotide pApA, were detected in the course of phosphorolysis. This was further confirmed by Godefroy (1970) with purified E. coli B polynucleotide phosphorylase.

3) Exchange

$$NDP + Pi \overset{Mg^{2+}}{\rightleftharpoons} NDP + Pi$$

There has been relatively little work published on the exchange reaction of polynucleotide phosphorylase as compared with the polymerization and phosphorolysis reactions. Early work (Grunberg-Manago, 1963) had shown that the exchange was specific for ribonucleoside diphosphate. The kinetics of the exchange reaction were very complex: the rate of exchange of ^{32}P-orthophosphate with each of the nucleoside diphosphates (ADP, GDP, UDP, CDP and IDP) was markedly dependent on the molar ratio of nucleoside diphosphate to orthophosphate and the optimal ratio was in turn dependent on the Mg^{2+} concentration. At a Mg^{2+} concentration of 5mM (generally used in the assay), the optimal molar ratio of nucleoside diphosphate to orthophosphate was about 1 for CDP and IDP, 0.7 for ADP, 2 for UDP and 0.17 for GDP (Grunberg-Manago, 1961). The reaction rates decreased sharply for values of the concentration ratio above or below the optimal ratio.

In a primer-dependent A. vinelandii polynucleotide phosphorylase preparation where the polymerization of ADP, UDP and CDP were greatly stimulated by oligonucleotides, the exchange reaction was only slightly stimulated (Singer, Hilmoe and Grunberg-Manago, 1960). Stimulation was observed with both oligonucleotides with free 3'-hydroxyl end and those with phosphate esterified at the 3'-end.

The M. lysodeikticus enzyme behaved somewhat differently: the exchange reaction could be stimulated slightly or considerably by oligonucleotides depending on the assay conditions used. Oligonucleotides with phosphate esterified at the 3'-end neither stimulated nor inhibited the exchange reaction (Singer and Guss, 1962). The polymerization of GDP under normal conditions required oligonucleotides for activity, but the GDP/Pi exchange reaction occurred readily in the absence of oligonucleotides if the appropriate GDP/Pi ratio was used. At the optimal GDP/Pi ratio, the rate of exchange of GDP with ^{32}P-orthophosphate occurred as readily as that found for other nucleoside diphosphates (Singer, Hilmoe and Grunberg-Manago, 1960).

Two hypotheses had been considered to explain the presence of the exchange reaction (Grunberg-Manago, 1963). In order to simplify the discussion, only the ADP/Pi exchange reaction will be considered. The two hypotheses were:

(i) the observed exchange reflects the reversible formation of a nucleoside monophosphate enzyme complex (E-AMP) (Olmstead, 1958);

(ii) the apparent exchange is a result of the occurrence of the overall reversible reaction (polymerization and phosphorolysis) under approximately equilibrium conditions.

These two hypotheses were discussed by Grunberg-Manago (1963). She favoured the second mechanism although there was not enough evidence at that time to eliminate the first entirely.

Recently, Kaufman and Littauer (1969) suggested that the exchange reaction was due to the formation of an enzyme-nucleoside

monophosphate complex (hypothesis (i)). They found that E. coli polynucleotide phosphorylase catalysed a slow dADP/Pi exchange reaction after prolonged incubation (20 hour at 37°C). The enzyme did not catalyse de novo polymerization of dADP but did catalyse a limited polymerization of dADP in the presence of oligonucleotide primer (ApA). The polymers obtained were mainly ApApdA and some ApApdApdA. The enzyme also catalysed the phosphorolysis of ApApdA to give ApA and dADP as the only product. From these observations they suggested that the exchange reaction was due to the formation of an enzyme-dAMP complex. They proposed that, by using ^{14}C-dADP, it should be possible to isolate the enzyme-dAMP complex.

The ability of polynucleotide phosphorylase to catalyse limited polymerization of dADP in the presence of oligonucleotide primer was confirmed by Bon, Godefroy and Grunberg-Manago (1970) with the E. coli enzyme and by Chou and Singer (1971a) with the M. lysodeikticus enzyme. However, the latter two groups of workers could not reproduce the dADP/Pi exchange reaction in the absence of oligonucleotide. They found that dADP/Pi exchange reaction required the presence of oligonucleotide for exchange. The dADP/Pi exchange reaction also occurred in the presence of small amounts of ADP but the rate of exchange was much slower than when oligonucleotide was present. Both Kaufman and Littauer (1969) and Bon, Godefroy and Grunberg-Manago (1970) obtained their dADP from the same supplier (Sigma). However, the latter group purified their dADP to remove any contaminating ADP present. Chou and Singer (1971a) obtained their dADP from a different sup-

plier (Calbiochem) and found that it was not necessary to purify their dADP from contaminating ADP. They did not obtain any exchange with dADP in the absence of oligonucleotide and assumed that there was no ADP contamination in their dADP. They found that as little as 5% contamination of the dADP by ADP was enough to catalyse complete equilibration between the terminal phosphate of dADP with ^{32}P-orthophosphate in the presence of M. lysodeikticus polynucleotide phosphorylase. From this observation, they suggested that the dADP used by Kaufman and Littauer was contaminated by small amounts of ADP. dADP inhibits ADP/Pi exchange, polymerization of ribonucleoside diphosphate and the phosphorolysis reaction (Bon, Godefroy and Grunberg-Manago, 1970; Chou and Singer, 1971a,b,c).

To summarize the above discussion: polynucleotide phosphorylase catalysed limited addition of dAMP units into oligonucleotide primer. It could catalyse phosphorolysis of ApApdA to give ApA and dADP. It could only catalyse exchange between dADP and orthophosphate in the presence of oligonucleotide primer. The ability of the enzyme to catalyse exchange in the presence of ADP is explained by assuming that the enzyme synthesized a small amount of oligoribonucleotide and that dADP was added to this primer. In the presence of high concentrations of orthophosphate, the ribonucleotide deoxyribonucleotide copolymer was then phosphorolysed. This mechanism could also explain the ability to catalyse dADP/Pi exchange in the presence of oligonucleotide, (Bon, Godefroy and Grunberg-Manago, 1970; Chou and Singer, 1971a).

Simuth et al (1971), who studied the reactions of 2'-O-methyl-cytidine-5'-diphosphate as substrate for E. coli polynucleotide phosphorylase, came to the same conclusion, i.e. that the exchange reaction was due to the overall reversible polymerization and phosphorolysis reaction operating under equilibrium conditions. Chou and Singer (1971a) had attempted to demonstrate the presence of an enzyme-dAMP complex under various conditions and had failed to do so. The hypothesis that the exchange reaction did not involve an enzyme-nucleoside monophosphate complex was further strengthened by the fact that one of the E. coli Q13 polynucleotide phosphorylases and the enzyme from the E. coli PR7 mutant that lack polymerization activity also lack nucleoside diphosphate-orthophosphate exchange activity. These enzymes had phosphorolytic activity only (Thang, Thang and Grunberg-Manago, 1969; Reiner, 1969b).

4) The transnucleotidation reaction

$$pApApA + pApApA \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} pApApApA + pApA$$

This reaction was detected by Singer et al. (1959) in a partially purified A. vinelandii polynucleotide phosphorylase preparation. When they incubated oligonucleotides in the presence of Mg^{2+}, they observed the formation of longer chain oligonucleotides, without any release of ADP or inorganic phosphate. There was no further publication dealing with this reaction since this initial report by Singer et al. (1959). The ability of A. vinelandii polynucleotide phosphorylase to catalyse transnucleotidation has been confirmed (Gajda and Fitt, unpublished results) with a highly

purified A. vinelandii preparation, but the lack of a convenient assay system has prevented any detailed study of the reaction.

5) Properties of polynucleotide phosphorylases from E. coli mutants

The first polynucleotide phosphorylase to be isolated from an E. coli mutant was obtained from E. coli Q13. This mutant was originally isolated in W. Gilbert's laboratory by the action of nitrosoguanidine on E. coli A19. It was reported that E. coli Q13 (Hfr, methionine$^-$, tyrosine$^-$, RNase I$^-$, polynucleotide phosphorylase$^-$) had no detectable polynucleotide phosphorylase (W. Gilbert, quoted by Hsieh and Buchanan, 1967; Haruna and Speigelman, 1965; Ben-Hamida and Schlessinger, 1966). Natori and Mizuno (1967) studied the degradation of pulse labelled RNA in E. coli Q13 ribosomal preparation[5] and found that RNA degradation was stimulated by the presence of K_2HPO_4. When they separated the product of degradation of the pulse labelled RNA in the presence of phosphate, they detected large quantities of nucleoside monophosphates but also small amounts of nucleoside diphosphates. They could not detect any activity in the crude extract when ADP/Pi exchange was used for the assay for polynucleotide phosphorylase. They also found that all the added ADP in the reaction was converted to AMP. The nucleoside diphosphate pyrophosphatase activity was found to be very high, four times higher than that found in the wild strain E. coli. From these observations they suggested that polynucleotide phosphorylase was present in E. coli Q13, but that its activity was masked by the high pyrophosphatase activity.

Hsieh and Buchanan (1967) isolated and partially purified a polynucleotide phosphorylase from E. coli Q13. They showed

that this enzyme catalysed polymerization of ADP, UDP and CDP in the presence of Mn^{2+}; Mg^{2+} could not replace Mn^{2+}. There was negligible phosphorolytic or exchange reaction either in the presence of Mg^{2+} or Mn^{2+}. Thang, Thang and Grunberg-Manago (1967, 1969) also purified polynucleotide phosphorylase from E. coli Q13 and compared its properties with the enzymes from E. coli B and E. coli K12. They found that E. coli Q13 contained two types of polynucleotide phosphorylase. One enzyme had molecular weight of 200,000 which is similar to the molecular weight of E. coli B and E. coli K12 enzymes. However, its properties were different from those of the "normal" E. coli enzyme. The polymerization reaction required Mn^{2+} for activity as shown by Hsieh and Buchanan (1967) but, it also catalysed a Mg^{2+} dependent phosphorolysis of poly A. The K_m for poly A was much higher than that of the "normal" E. coli strains. This mutant also contained a lower molecular weight species of polynucleotide phosphorylase. This enzyme had a molecular weight of 100,000, which is half of that of the "normal" E. coli strains' enzyme. This low molecular weight enzyme only catalysed the phosphorolytic reaction. This reaction required high Mg^{2+} concentration and phosphorolysed short chain oligonucleotides only.

Castles and Singer (1968) studied the properties of polynucleotide phosphorylase purified from E. coli 1113B. This mutant was derived from E. coli Q13 but differs from E. coli Q13 in that it has a heat-sensitive ribonuclease II. They separated the E. coli 1113B polynucleotide phosphorylase into two enzymes by Sephadex G-200 gel filtration. The two enzymes catalysed the

polymerization reaction. The larger molecular weight enzyme was Mn^{2+} dependent and its activity was reduced by 90% when Mg^{2+} replaced Mn^{2+}. The lower molecular weight enzyme was equally active with Mn^{2+} or with Mg^{2+}. Sucrose density gradient centrifugation indicated that the higher molecular species was twice as large as the smaller molecular species. This was in agreement with the results obtained by Thang, Thang and Grunberg-Manago (1969) with E. coli Q13. However, the latter authors reported that their smaller molecular weight enzyme did not catalyse the polymerization reaction. The two polynucleotide phosphorylases obtained from E. coli 1113B catalysed only a very limited phosphorolysis of poly A. Polynucleotide phosphorylase was also studied in other E. coli mutants by Reiner (1969a,b). He confirmed the presence of Mn^{2+} dependent polymerization activity of E. coli Q13 polynucleotide phosphorylase, but he could not detect the low molecular weight species. He used high molecular weight poly A to detect phosphorolysis, but Thang, Thang and Grunberg-Manago (1969) showed that this low molecular weight enzyme only phosphorolysed oligonucleotides and not high molecular weight poly A. Reiner (1969a,b) also reported that the E. coli PR7 mutant had a low phosphorolysis activity, but lacked polymerization or exchange activities. This enzyme phosphorolysed high molecular weight poly A. All the other mutants studied had low and altered polynucleotide phosphorylase activity.

6) Summary on the specificity of polynucleotide phosphorylase and its importance in the detection of the presence of the enzyme.

The specificity of bacterial polynucleotide phosphorylase

has been discussed in detail by Grunberg-Manago (1963). In general, the polymerization reaction is specific for ribonucleoside diphosphate: ribonucleoside tri- and mono-phosphates are not substrates. The specificity of the enzyme for ribonucleoside diphosphate is not absolute since it can catalyse a limited incorporation of dADP in the presence of an oligonucleotide primer. The rate for this reaction is very slow as compared with that for the polymerization of the ADP. This ability to incorporate the deoxy analogue into the polymer is not unique to polynucleotide phosphorylase. Yeast glycogen synthetase had been shown to catalyse incorporation of 2-deoxy-D-glucose into glycogen (Zemek et al, 1971). The deoxy analogue of UDPG, UDPDG, can also serve as 2-deoxy glucosyl donor in the biosynthesis of 2-deoxy sucrose, 2,2'-dideoxy trehalose (quoted by Zemek et al, 1971).

The phosphorolytic reaction is specific for polyribonucleotides and neither native nor denatured DNA are phosphorolysed. The product of phosphorolysis are ribonucleoside diphosphates only (with the exception of the final dinucleotide). The rate of phosphorolysis of single stranded polynucleotides is much greater than that for the phosphorolysis of polynucleotide with secondary structures. The rate decreases as the amount of secondary structure in the polymer increases.

Both the polymerization and phosphorolysis can be used to demonstrate the presence of polynucleotide phosphorylase.

If the polymerization reaction is used, it has to be demonstrated that the polymerization is specific for nucleoside diphosphates rather than the triphosphates, and that the systems examined do not contain high activities of enzymes that convert

nucleoside diphosphates or monophosphates to nucleoside triphosphates. There have been many reports in the literature where enzyme preparations, from many sources, could catalyse the polymerization of single ribonucleoside triphosphate species (Smellie, 1963; Grunberg-Manago, 1961, 1963; Hurwitz and August, 1963). Partially purified RNA polymerase from E. coli and M. lysodeikticus catalysed synthesis of poly A from ATP in the presence of denatured DNA (Chamberlin and Berg, 1964; Stevens, 1964; Fox and Weiss, 1964), or polyribonucleotides as template (Stevens, 1964; Niyogi and Stevens, 1965). A number of less well defined enzyme preparations which could polymerize ATP, UTP or CTP in the presence of RNA or oligonucleotide primers have been described (Hurwitz and Bresler, 1961; Edmonds and Abrams, 1962; Edmonds, 1965; August, Ortiz and Hurwitz, 1962; Wilkie and Smellie, 1968; Kato and Kurokawa, 1970; Dravid, Pete and Mandel, 1971; Twu and Bretthauer, 1971).

If the phosphorolysis reaction is used to demonstrate the presence of polynucleotide phosphorylase, it has to be shown that the products formed are nucleoside diphosphates and that little or no nucleoside triphosphate is formed. In a crude enzyme preparation, any system that can generate a large amount of nucleoside triphosphate will be able to form nucleoside diphosphate from nucleoside 5'-monophosphate present in the extract or produced by an exo-ribonuclease action on polyribonucleotides. Thus, in order to demonstrate the presence of polynucleotide phosphorylase by the phosphorolysis reaction, it had to be shown that little or no nucleoside triphosphate is formed during the reaction.

The exchange reaction catalysed by polynucleotide phosphorylase is specific for ribonucleoside diphosphates, and nucleoside tri- or mono-phosphates cannot act as substrates. However this exchange reaction is not specific to polynucleotide phosphorylase. Grunberg-Manago et al. (1966) isolated an enzyme from yeast that catalysed exchange of orthophosphate with ribonucleoside diphosphates, deoxyribonucleoside diphosphates and adenosine phosphosulphate. This enzyme did not catalyse the polymerization of ribonucleoside diphosphates nor the phosphorolysis of polyribonucleotides. Stern and Avron (1966) also isolated an enzyme (Adenosine 5'-diphosphate ribose: orthophosphate adenyltransferase) from an extract of Euglena gracilis which catalysed ADP/Pi exchange. This exchange is specific for ADP and IDP; ATP, AMP, CDP, GDP and UDP were not substrates. The enzyme also catalysed the phosphorolysis of adenosine diphospho-5'-ribose (ADPR) to ADP and ribose-5'-phosphate:

$$\text{ADPR} + P_i \overset{Mg^{2+}}{\rightleftharpoons} \text{ADP} + \text{ribose-5'-phosphate}$$

Since the exchange reaction is not specific to polynucleotide phosphorylase, this reaction, alone, cannot be used to demonstrate the presence of polynucleotide phosphorylase.

B. Animal Polynucleotide Phosphorylase

Polynucleotide phosphorylase-like activities have been detected in a number of animal tissues preparations: guinea pig liver nuclei (Hilmoe and Heppel, 1957), particulate fraction of reproductive tract of Ascaris lumbricoides (Entner and Gonzalez, 1959), human sperm and urine (Hakim, 1959a,b), rat epithelioma (Yagi, Ogawa and Konogi, 1959), Hela cell nuclei (Harris, 1963), rat

liver nuclei (Siebert et al, 1966) and in various fractions of rat liver and brain homogenates (Murthy, Bharucha and Raynaud-Jammet, 1969). The publications dealing with animal polynucleotide phosphorylase prior to 1963 have been discussed and questioned by Smellie (1963). Smellie's criticism can be applied equally well to more recent references given above.

The first report of the presence of polynucleotide phosphorylase in animal tissue was a preliminary note published by Hilmoe and Heppel (1957). They showed that an extract from preparation of guinea pig nuclei could catalyse phosphorolysis of poly A in the presence of ^{32}P-labelled orthophosphate. The products obtained were ATP, ADP and AMP. It is significant to point out that there was as much radioactivity incorporated into ATP as into ADP and the specific radioactivity of the ATP was much higher than that of the ADP. Since there was a large amount of ATP produced, other reactions could be postulated to account for the formation of labelled ADP (Smellie, 1963). In the presence of ribonuclease that could degrade poly A to AMP and a kinase system that could generate ATP, ADP could be produced by adenylate kinase. In this connection an exoribonuclease that degrades poly A to 5'-AMP has been extracted and purified from rat liver nuclei (Lazarus and Sporn, 1967; Sporn et al, 1969). Hilmoe and Heppel did not give any details of how their nuclei and nuclear extracts were obtained, and published no further work on this topic, so it is impossible to assess their data any further.

Entner and Gonzalez (1959) also published a preliminary note on the presence of polynucleotide phosphorylase in a particulate

fraction from Ascaris lumbricoides reproductive tract. The authors detected phosphorolysis of poly A and poly C and stated that the products were ADP and CDP exclusively. They also detected polymerization of CDP and CTP. However insufficient details of the preparation of the particulate fraction were given by the authors to permit meaningful discussion. Smellie (1965) assumed that these particles contained both microsomes and mitochondria and the same criticisms can be made of these data as in the case of the work of Hilmoe and Heppel (1959). Entner and Gonzalez also stated that further work was in progress on this topic, but no further paper was ever published.

The data indicating that polynucleotide phosphorylase might be present in rat epithelioma (Yagi, Ozawa and Konogi, 1959) and in human sperm and urine (Hakim, 1959a,b) have been discussed in detail by Smellie (1963) and will not be discussed in detail here. I will only mention that there was insufficient data given by Yagi et al. for any critical assessment to be made. Hakim did not mention what precautions were taken in collecting his samples, and it is possible that what he observed was due to bacterial contaminations.

Harris (1963) found that the degradation of pulse-labelled RNA by isolated Hela cell nuclei in the presence of orthophosphate produced small amounts of nucleoside diphosphates and large amounts of nucleoside monophosphates. When the nuclei were solubilized in 1M NaCl and then diluted to 0.14M sodium chloride with water, a crude chromatin material could be isolated and contained the pulse-labelled RNA. The degradation of this labelled RNA in

the presence of phosphate also produced a large amount of nucleoside monophosphates and a small amount of nucleoside diphosphates. He suggested that the degradation of the pulse-labelled RNA (rapidly-labelled RNA) was due to polynucleotide phosphorylase. The presence of strong phosphatases degraded the nucleoside diphosphates to nucleoside monophosphates. Siebert et al. (1966) studied the degradation of added RNA in the presence of orthophosphate and suggested that polynucleotide phosphorylase was present in both the nuclei and the nucleoli of rat liver. The production of nucleoside diphosphates was measured by the oxidation of NADH in the presence of a coupled enzyme system (phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase). However, this method for the assay of polynucleotide phosphorylase in whole nuclei or nucleoli was unreliable. There was a considerable drop in absorbance at 340nm in the absence of NADH and the coupled system (Siebert and Kesserling, 1966; Pennial et al., 1968). Pennial et al. (1968) stated that "spectrophotometric assays (using the above system for assay) of polynucleotide phosphorylase in nucleoli either with or without exogenous RNA has been equivocal or negative". Murthy, Bharucha and Raynaud-Jammet (1969) suggested that polynucleotide phosphorylase was present in the soluble fraction of rat liver and rat brain homogenates. Their evidence was based on the fact that the degradation of ^{14}C-poly A was stimulated by the addition of inorganic phosphate. They also detected ADP/Pi exchange activity in crude liver and brain homogenates. No attempt was made to identify the products.

C. Plant Polynucleotide Phosphorylase

There have been two reports dealing with plant polynucleotide phosphorylases.

Brummond, Staehelin and Ochoa (1957) detected some nucleoside diphosphate-orthophosphate exchange activity in an ammonium sulphate fraction (30-60% ammonium sulphate) from a soluble extract of spinach leaves, but only very little evidence was presented and, as discussed above (p. 27) the exchange reaction is not specific to polynucleotide phosphorylase.

Kessler and Chen (1964) showed that wheat root subcellular fractions (nuclear, microsomal, mitochondrial and soluble) catalysed both polymerization of ^{14}C-nucleoside diphosphates into acid insoluble materials and ADP/Pi exchange. These authors devoted much effort to the study of the properties of the various fractions, but unfortunately no attempt was made to establish the substrate specificity of the polymerization activity. Thus, it still remains uncertain whether the enzymes studied by Kessler and Chen was really polynucleotide phosphorylase or a combination of enzymes which finally catalysed the polymerization of nucleoside triphosphates formed from the nucleoside diphosphates supplied as substrate.

D. Conclusion

Thus, although the work discussed above suggests that polynucleotide phosphorylase may be present in eucaryotic cells, no unequivocal evidence was presented by any of the authors concerned.

E. The Physiological Function of Polynucleotide Phosphorylase

The precise physiological role of polynucleotide phosphorylase

in the bacterial cell is still uncertain, but it is generally accepted that it is degradative. A number of possible functions have been suggested (Grunberg-Manago, 1963): (i) the control of the inorganic phosphate level in the cell; (ii) the production of ribonucleoside diphosphates as immediate precursors of the deoxyribonucleotides; (iii) the degradation of messenger RNA. No definite proof exist for any of these hypotheses, of which the third has been most favoured (Wade and Lovett, 1961; Sekiguchi and Cohen, 1963; Andoh, Natori and Mizuno, 1963; Kimhi and Littauer, 1967; Castles and Singer, 1969; and others: see Singer and Leder (1966) for additional references). The main stumbling block in this hypothesis is that m-RNA is now known to be degraded in a 5' to 3' direction (Morikawa and Imamoto, 1969; Morse et al., 1969a,b) while polynucleotide phosphorylase is known to degrade polyribonucleotides stepwise, from the 3' to 5' end (Hilmoe, 1959; Singer, Hilmoe and Grunberg-Manago, 1960; Thang et al., 1967; Klee and Singer, 1968; Godefroy, 1970).

F. Purpose of this Research Described in this Thesis

The work described in this thesis is part of a general study whose eventual aim is to discover the physiological role of polynucleotide phosphorylase. As a first approach to the problem, it is necessary to know how widely the enzyme is distributed among different organisms and to determine its exact location in the cell. It should be noted that its actual location in the bacterial cell is still uncertain (Grunberg-Manago, 1963); and is discussed in detail later in the thesis (p.192).

The specific aims of my research were: (i) to show that poly-

nucleotide phosphorylase is definitely present in animal cells; (ii) to study its location in animal cells; (iii) to purify the enzyme and study its properties in order to compare them with those of the bacterial enzymes; (iv) to determine how widely polynucleotide phosphorylase is distributed in animal tissues and among animal species.

II. LOCALIZATION, PARTIAL PURIFICATION AND DISTRIBUTION OF ANIMAL POLYNUCLEOTIDE PHOSPHORYLASE

Materials and Methods

A. Materials

Animals, chemicals and intermediates were purchased from the following suppliers: ^{14}C-nucleoside mono-, di- and triphosphates and ^{32}P-orthophosphate: Amersham/Searle Corp., Don Mills, Ont., Canada.

^{14}C-poly A: Schwarz Bioresearch, Inc., Orangeburg, N.Y., U.S.A.

Unlabelled nucleoside mono-, di- and triphosphates: P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.

dADP and high molecular-weight wheat-germ RNA (rRNA): Calbiochem, Los Angeles, Calif., U.S.A.

Oligonucleotides and Serva TLC-DEAE-cellulose: Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y., U.S.A.

Poly A, poly U, poly C and poly G: Miles Chemical Co., Elkhart, Ind., U.S.A.

Torula yeast RNA (grade VI), Calf thymus DNA, cytochrome C (horse heart, grade VI), Tris(hydroxymethyl)aminomethane, disodium succinate, NADH: - Sigma Chemical Co., St. Louis, Mo., U.S.A.

DNase I: Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Bovine serum albumin: Mann Research Laboratories, New York, U.S.A.

Phospholipid standards: Cardiolipin (beef heart), L-α-lysophosphatidylcholine, phosphatidylcholine, and phosphatidylethanolamine: General Biochemicals, Chagrin Falls, Ohio, U.S.A.

Sodium dodecyl sulphate (specially purified grade): B.D.H. (Canada) Ltd., Toronto, Ont., Canada.

2-Mercaptoethanol: Eastman Kodak Co., Rochester, N.Y., U.S.A.

Triton X-100: Hartman-Leddon Co., Philadelphia, Pa., U.S.A.

Digitonin: Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Whatman DE23 DEAE cellulose, Reeve Angel: Clifton, N.J., U.S.A.

Other chemicals: Fischer Scientific Co., Ottawa, Ont. Canada.

Sprague-Dawley rats: Bio-Breeding Laboratories, Ottawa, Ont. Canada.

Guinea-pigs: Robidoux, St. Constant, P.Q., Canada.

Rabbits: University of Ottawa animal house.

Roosters and bovine liver: local suppliers.

Gold fish were a gift from Dr. J.C. Fenwick, Biology Department, University of Ottawa.

II. B. Methods

The pH of buffers refers to the value at 25° and all temperatures are in °C.

(i) Enzyme assays

Phosphorolysis

Polynucleotide phosphorylase activity was determined by the phosphorolysis assay, which depends on the formation of ^{32}P-NDP during phosphorolysis of polyribonucleotides in the presence of ^{32}P-orthophosphate (Grunberg-Manago, Ortiz and Ochoa, 1956; Fitt and Fitt, 1967). The standard assay medium for rat liver mitochondrial enzyme (0.1 ml) contained: 0.1M-Tris-HCl buffer, pH 8.2 (or 7.8, if used for the membrane-associated enzyme); 6mM-$MgCl_2$; 1mM-2-mercaptoethanol; 10mM-$K_2H^{32}PO_4$ (50000-100000 c.p.m./µmol.); poly A, 1.5 mg/ml; 0.1% (v/v) Triton X-100; enzyme. The Triton X-100 was omitted for the assay of rat liver nuclear enzyme. Incubation was at 37° for 1 h in a water bath shaker. The reaction was stopped with 1 ml of a mixture containing 1 part of an aqueous 10% (w/v) suspension of acid washed Norit A and 10 parts of 2.5% (w/v) $HClO_4$. The charcoal suspension was left for 10 minutes at 0° and was then collected on a 2.4 cm diameter Whatman GF/C glass fibre filter. The filter was washed four times with 10 ml of water and was then inverted in a planchet, dried and its radioactivity determined in a Nuclear-Chicago low-background thin-window counter. The specific radioactivity of the $K_2H^{32}PO_4$ was determined by counting a dried portion of the solution over which a GF/C filter had been placed. Controls without enzyme were carried out in all

cases and their radioactivities subtracted from the assay values. A unit was defined as the amount of enzyme catalysing incorporation of 1 nmol of ^{32}P-P_i into charcoal-adsorbable material/h.

Guinea-pig liver nuclear polynucleotide phosphorylase was assayed as described above, in the absence of Triton X-100 except that 0.1M-tris-HCl buffer, pH 8.7 and 4mM $MgCl_2$ was used.

NDP-P_i exchange activity was assayed by measuring the incorporation of ^{32}P-P_i into nucleoside diphosphates (Grunberg-Manago, Ortiz and Ochoa, 1956). (i) Rat liver mitochondrial enzyme. The reaction mixture (0.1 ml) contained: 0.1M-tris-HCl, pH 7.8; 6mM $MgCl_2$; 1mM-2-Mercaptoethanol; 10mM-$K_2H^{32}PO_4$ (50000-100000 c.p.m./µmole); 5mM-NDP; 0.1% (v/v) Triton X-100; enzyme. (ii) Guinea-pig nuclear enzyme. The reaction mixture (0.1 ml) contained: 0.1M Imidazole-HCl buffer, pH 7.8, 10mM-$MgCl_2$; 1mM-2-Mercaptoethanol; 10mM $K_2H^{32}PO_4$ (30000-100000 c.p.m./µmole); 1mM-NDP; enzyme. Incubation was at 37° for 1 hour in a water-bath shaker and the samples were processed as described for the phosphorolysis assay. Controls without substrates were carried out and their radioactivities subtracted from the assay values. The amount of ^{32}P-orthophosphate incorporated into charcoal-adsorbable material was calculated by the formula:

$$\text{nmol of } {}^{32}P \text{ incorporated} = \frac{\text{c.p.m. incorporated (nmol } P_i + \text{ nmol NDP)}}{\text{c.p.m. in reaction}}$$

A unit was defined as the amount of enzyme catalysing incorporation of 1nmol of ^{32}P-P_i into charcoal-adsorbable material/h.

NTP-Pi exchange activity was assayed by measuring the incorporation of ^{32}P-P_i into nucleoside triphosphates. The conditions and procedure were the same as for the NDP-P_i exchange assay with NTP substituted for NDP. For assay of the guinea-pig nuclear enzyme, imidazole-HCl buffer pH 7.4 was used.

Polymerization of nucleoside diphosphates. This assay depends on the polymerization of ^{14}C-nucleoside diphosphate into an acid-insoluble form (Littauer and Kornberg, 1957; Fitt and Fitt, 1967). The reaction mixture (0.1ml) contained: 0.1M-tris-HCl buffer, pH 7.8; 6mM $MgCl_2$; 1mM-2-mercaptoethanol; 5mM-^{14}C-nucleoside diphosphate (about 170000 c.p.m./μmol); 0.1% (v/v) Triton X-100; enzyme. Incubation was at 37° for 1 hour in a water bath shaker. The reaction was stopped with 0.1ml of 7% (w/v) $HClO_4$ and the mixture was kept at 0°C for 10 minutes. The precipitate was collected on a Whatman GF/C glass-fibre filter, washed four times with 1% (w/v) $HClO_4$ (2ml) and once with 50% (v/v) ethanol (2ml), dried and the radioactivity counted in a Nuclear-Chicago low-background thin window counter. Controls lacking enzyme were performed in a similar way and the radioactivity subtracted from the assay value.

Ribonuclease activity was determined by measuring acid soluble radioactivity released during hydrolysis of ^{14}C-poly A. The reaction mixture (0.1ml) contained: 0.1M-tris-HCl

buffer, pH 7.8; 6mM-$MgCl_2$; 1mM-2-mercaptoethanol; ^{14}C-poly A, 1.5 mg/ml (36000 c.p.m./mg); 0.1% (v/v) Triton X-100. Incubation was at 37^{o} for 1 h in a water bath shaker and the reaction was stopped by addition of 7% (w/v) $HClO_4$ (0.1ml). The mixture was kept in ice for 10 min and then centrifuged in a clinical centrifuge at room temperature. Samples (50µl) of the supernatant were dried on planchet and counted in a Nuclear-Chicago low-back ground thin-window counter. Controls lacking enzyme were performed in a similar way and their radioactivity subtracted from the assay value.

Adenylate kinase was detected qualitatively by the production of ^{14}C-ATP from ^{14}C-ADP. The assay mixtures and assay conditions were as given for the polymerization assay (see above) except 1mM ^{14}C-ADP was used. The reaction was stopped by transferring the tubes to an ice bath, the mixtures were kept at 0^{o} for 5 min, centrifuged in a clinical centrifuge at room temperature (2 min at full speed) and then kept at 0^{o}. Aliquots (25µl) were applied to a TLC plate coated with Serva TLC-DEAE-cellulose. The chromatogram was developed with 4% (w/v) formic acid (Fahn, Albers and Koval, 1965). The nucleotides were located with u.v. light, and the area corresponding to each nucleotide was scraped off and its radioactivity counted in a liquid scintillation counter. The DEAE-cellulose powder was added to 5ml of toluene base scintillation fluid containing 0.5% (w/v) PPO (2,5-diphenyloxazole) and 2% (v/v) ethanol. A control without enzyme was run exactly as above and the results were corrected accordingly.

Phosphatase assays. Two procedures were used for the determination of nucleoside diphosphatase activities:

(a) The conversion of ^{14}C-ADP to ^{14}C-AMP. This procedure was used as a sensitive and qualitative assay for the detection of ADPase activity. The difference between the count in ^{14}C-AMP and ^{14}C-ATP was taken to represent the hydrolysis of ^{14}C-ADP by ADPase. The conditions and procedures for the assay were as described for the adenylate kinase assay.

(b) Nucleoside diphosphatase activity was assayed by estimation of the inorganic phosphate released during the hydrolysis of nucleoside diphosphates. The reaction mixture (1ml) contained: 0.1M-tris-HCl buffer, pH 8.0; 5mM-$MgCl_2$; 1mM-2-mercaptoethanol; 1mM-nucleoside diphosphate; 0.1% (v/v) Triton X-100; enzyme. Incubation was at 37^{o} for 30 min in a water bath-shaker. The reaction was stopped with cold 6% (w/v) $HClO_4$ (1ml) and the orthophosphate released was determined by one of the procedures described in section III (procedures developed for the determination of inorganic phosphate in the presence of Triton X-100). A unit of phosphatase activity was defined as the amount of enzyme catalysing the release of 1µmol of Pi/30 min.

Urate oxidase was measured by the procedure described by Schneider and Hogeboom (1952). The rate of oxidation of uric acid was followed by measuring the decrease in extinction at 290nm. The molar extinction coefficient at 290nm and pH 7.0 ($E \times 10^{-4} = 1.22$) as determined by Kalckar (1947) was used for calculation. The stock of uric acid solution (12.5mM) was prepared,

fresh just before use , by dissolving uric acid in 0.025N NaOH. The reaction mixture (1ml) contained: 0.033M potassium phosphate buffer, pH 7.4; 42μM uric acid; 0.1% Triton X-100 and enzyme. The reaction was followed spectrophotometrically at 37°. A unit of activity was defined as the amount of enzyme catalysing the oxidation of 1μmol. uric acid/min. The use of 0.1% (v/v) Triton X-100 in this assay was first described by Beaufay et al. (1959).

Acid phosphatase was determined by the procedure of Linhardtand Walter (1963) in the presence of Triton X-100 (Vignais and Nachbaur, 1968). A stock solution of 5.5mM p-nitrophenyl phosphate in 50mM citrate buffer, pH 4.8 was prepared just before use. The reaction conditions were as follows: a mixture of 0.3ml stock substrate solution and 0.03ml 1% (v/v) Triton X-100 was equilibrated at 37° for 5 min. At zero time, 0.02ml of enzyme solution was added to start the reaction. Incubation was at 37° for 30 min. and the reaction was stopped by the addition of 1ml of 0.1N NaOH. The yellow colour formed was measured at 400nm against a control performed without enzyme. A unit of activity was defined as the amount of enzyme catalysing the hydrolysis of 1nmol of p-nitrophenyl phosphate/h.

Cytochrome oxidase was measured by the procedure described by Wharton and Tzagoloff (1967).

(a) Preparation of reduced cytochrome c. Potassium ascorbate was added to a 1% (w/v) solution of cytochrome c (in 10mM potassium phosphate buffer, pH 7.0) until the solution was light pink in colour and further addition of ascorbate did not produce a visible change in colour. The excess ascorbate was removed by dialysis

against 100 vol. of 10mM potassium phosphate buffer, pH 7.0 for 8 h (4 changes of buffer). The reduced cytochrome c was stored at -20°.

(b) Cytochrome oxidase activity was assayed spectrophotometrically at 37° by measuring the decrease in extinction at 550nm. All reagents, except the enzyme solution, were pre-equilibrated at 37°. The reaction mixture (1ml) contained: 10mM potassium phosphate buffer pH 7.0; 0.1% (w/v) reduced cyt. c; 0.1% (v/v) Triton X-100 and enzyme (10µl of appropriate dilutions). The control contained potassium ferricyanide (10µl of 0.1M solution) instead of the enzyme. A unit of activity was defined as the amount of enzyme catalysing the oxidation of 1µmol cytochrome c/min.

Rotenone-insensitive NADH-cytochrome c reductase was assayed according to Sottocasa et al. (1967) and Schnaitman and Greenawalt (1968) by following, spectrophotometrically, the increase in extinction at 550nm due to the reduction of cytochrome c, at 37°. The reaction mixture (1ml) contained: 50mM potassium phosphate buffer, pH 7.4; 0.1mM NADH; 0.1mM cytochrome c; 0.3mM KCN; 5µM of rotenone; and enzyme. The extinction was measured against a control without enzyme. A unit of activity was defined as the amount of enzyme catalysing the reduction of 1µmol of cytochrome c/min.

Succinate-cytochrome c reductase was assayed as described by Sottocasa et al. (1967) at 37°. The reaction mixture contained (1ml): 50mM potassium phosphate buffer, pH 7.4; 3mM disodium succinate; 0.1mM cytochrome c; 0.3mM KCN; and enzyme.

The reaction was measured against a control without enzyme. The conditions and procedures for this assay were as described for the rotenone-insensitive NADH-cytochrome c reductase assay. A unit of activity was defined as the amount of enzyme catalysing the reduction of 1μmol of cytochrome c/min.

(ii) Phospholipid analysis. A suspension of enzyme particles (0.5 ml) was shaken for 20 min. in a stoppered glass centrifuge tube with 6 ml of chloroform-methanol (2:1 v/v) Folch et al., 1957). The mixture was centrifuged and the clear chloroform-methanol layer removed and evaporated to dryness in a rotary evaporator. The residue was dissolved in a few drops of chloroform and applied to a TLC plate coated with activated (110^{o} for 2 h) silica gel G. The chromatogram was developed with chloroform-methanol-water (65:25:4, by vol.) (Wagner, Horhammer and Wolff,1961). The lipids were located by either exposure to iodine vapour or sprayed with 50% (v/v) H_2SO_4 and heated in an oven at 110^{o} .

(iii) Nucleic acid determination. DNA was extracted and determined as described by Burton (1956), with calf thymus DNA as standard.

RNA was determined by the orcinol reaction, using the procedure of Dische (1955). The difference in the extinction of the test solutions at 675nm and 575nm was used for calculation and the internal glucose standard was omitted. A standard curve was determined with wheat germ RNA.

(iv) Protein was determined by the method of Lowry et al. (1951) in the presence of sodium dodecyl sulphate. Whole mitochondrial inner membranes or the particulate enzyme were first

dissolved by the addition of sodium dodecyl sulphate to a final concentration of 5mM. The blanks contained the same amount of buffer and detergent as used for the protein determination. A standard curve was determined with bovine serum albumin, the same concentration of buffer and detergent being used as were present in the enzyme samples. The stock bovine serum albumin solution (1mg/ml) was prepared by dissolving the solid in distilled water and the albumin concentration was determined from the ratio of the extinctions of the solution at 280nm and 260nm (Warburg and Christian, 1942; Layne, 1957).

(v) Determination of ADP contamination in dADP sample.

The amount of ADP present in commercial dADP was determined by paper chromatography. 5-20µl (0.25-1µmol) of a dADP solution (50µmol/ml) were applied to a Whatman No. 1 chromatographic paper under a stream of cold air. The paper was developed by descending chromatography for 75 h in ethanol/1M ammonium acetate, pH 9.0 (7.3, v/v) saturated with sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2$) (Klenow and Lichtler, 1957). The paper was dried and the nucleotides located under u.v. light. The areas corresponding to ADP were removed, cut into small pieces and the nucleotide was eluted at 4° with 0.1N HCl (1ml). The eluates were centrifuged and the E_{257} of the supernatants read against a control obtained by eluting a similar blank area of the chromatogram. The amount of ADP in each area was calculated and the % contamination was determined.

Methods for preparation of organelles, sub-cellular fractions and purification of enzymes.

All low speed centrifugations (600-40000 g) were performed in the International refrigerated centrifuge and high speed centrifugations in the Beckman L2-65B preparative ultracentrifuge. Unless otherwise stated, all operations were carried out at 0-4°.

(vi) The separation of peroxisomes, mitochondria and lysosomes in crude mitochondrial preparation.

Rat liver mitochondria were isolated by the method of Schnaitman and Greenawalt (1968). Two male rats (400-450 g) were decapitated and bled. The livers were removed and chilled in 100 ml ice cold 0.25M sucrose-10mM tris-HCl buffer, pH 7.4 (sucrose-tris buffer). The tissue was weighed, cut into small pieces and homogenized in a Dounce homogenizer with a loose fitting pestle (5 up and down strokes). The homogenate was diluted to 10 vol. with sucrose-tris buffer and centrifuged at 600 g for 15 min. The pellet was discarded and the supernatant was centrifuged at 7,000 g for 15 min. to sediment mitochondria. The mitochondrial pellet was washed twice with sucrose-tris buffer (0.5 and 0.25 respectively, of the original vol.). The washed mitochondria were resuspended in 10 ml of sucrose-tris buffer.

Sucrose-glycogen gradient centrifugation

The gradient Gh(10) of Beaufay et al. (1964) was prepared. The concentrations of sucrose and glycogen used are shown in the table below.

Solvent	Solution	Sucrose ($g/100g\ H_2O$)	Glycogen ($g/100g\ H_2O$)	Density
H_2O	light	19.2	0	1.068
	heavy	19.2	30.6	1.42

The light solution was prepared by dissolving 1.92 g of sucrose in 10 g of water.

The heavy solution was prepared by the following procedure:

3.06 g of oyster glycogen was weighed into a 20ml beaker. 5ml of water was added and the suspension was stirred with a glass rod until a homogenous paste was produced. Water was added (0.5ml at a time) and the suspension stirred with a glass rod, until a total of about 8ml of water was added. The suspension was stirred until all the glycogen had dissolved to give a greenish yellow solution. This process took about 3 hours. The pH of the solution was brought to pH 7.4 with 1M NaOH and water was added to give a total weight of 13.06 g. Solid sucrose (1.92 g) was added and stirred until dissolved. The sucrose-glycogen gradient (3.4ml) was prepared with a Buchler gradient-former (Buchler Instruments, Fort Lee, New Jersey U.S.A.). The prepared gradient was stored at 4^{o} for about 16 h (for equilibration) and 0.15ml of the mitochondria (freshly prepared) was then layered over it and centrifugation was at 40,000 rpm (157,000 g) for 2.25 h in a Beckman SW56 rotor. The centrifuge tubes were cut at 0.45 cm intervals (average vol/fraction: 0.22ml) using a tube slicer (Model I-3600, Microchemical specialities Co., Berkeley, Calif. U.S.A.). (N.B.: it is important to make sure that the knife of the tube slicer is well sharpened before attempting to cut the tubes. This can be checked using a similar tube filled with water.) The various fractions were transferred to numbered assay tubes with a Pasteur pipette. The pellet left at the bottom of the tube was suspended in 0.2ml sucrose-tris buffer.

(vii) Fractionation of mitochondria

Mitochondria were fractionated into outer membrane, inter-

membrane space and inner-membrane matrix fraction as described by Schnaitman and Greenawalt (1967, 1968) using 1.1mg digitonin/10mg mitochondrial protein. Inner membrane was prepared from the inner membrane-matrix fraction asdescribed by Beattie (1969) and See and Fitt (1971).

The washed mitochondria (10ml) prepared as described above (section (vi)) were collected by centrifugation at 10,000 g for 10 min. The pellet was resuspended in sucrose-tris buffer to give 100mg protein/ml. Digitonin (1% (w/v) stock solution in sucrose-tris buffer, prepared the same day) was added to give a ratio of 1.1mg digitonin/10mg protein. The resulting suspension was gently stirred for 15 min and then diluted with 3 vol. of sucrose-tris buffer. The diluted suspension was homogenized (3 strokes) in a loose fitting Dounce homogenizer and centrifuged at 12,000 g for 15 min. The supernatant was drawn off carefully (with a Pasteur pipette) and kept. The pellet was resuspended in the same volume of buffer and centrifuged at 12,000 g for 10 min. The supernatant was collected as before and the two supernatants were pooled (low speed supernatant). The pellet was the inner-membrane matrix fraction. The low speed supernatant was centrifuged at 40,000 g for 10 min to remove inner membrane fragments and then at 30,000 rpm (78,000 g) in a Beckman type 30 rotor for 90 min and the supernatant (solution of inter-membrane space) was collected. The pellet (outer membrane) was transferred to a small homogenizer (capacity 3ml) with sucrose-tris buffer (1.5 ml) and suspended by homogenization.

The inner-membrane matrix pellet was resuspended in 10ml of sucrose-tris buffer and 1ml was kept for enzyme assays. The remainder was sonicated for 30 sec with a Bronwill Bio-sonic III sonic disintegrator (small probe, dial setting 20) and centrifuged at 30,000 r.p.m. (78,000 g) in a Beckman type 30 rotor for 90 min. The supernatant (matrix fraction) was collected. The surface of the tightly packed pellet was rinsed with buffer (1 ml per tube) and the solid was then transferred to a Dounce homogenizer with sucrose-tris buffer (5ml) and resuspended to give the inner-membrane fraction.

(viii) Partial purification of rat liver mitochondrial polynucleotide phosphorylase

The procedures used for the isolation of mitochondria and inner membrane was slightly different from that of sections (v) and (vi) and will be described in full here.

a) Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by the method of Schnaitman and Greenalwalt (1968) with minor modifications. Six male rats (400-450 g) were decapitated and bled. The livers were removed and chilled in 100ml of ice-cold 0.25M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (sucrose-tris-ME buffer). The tissue was weighed, cut into small pieces and passed through a coarse sieve tissue press (Harvard Apparatus Co., Inc., Millis, Mass., U.S.A.) to remove connective tissue. It was then homogenized in sucrose-tris-ME buffer (2 vol.) with a motor-driven Potter-Elvehjem homogenizer (5 up-and-down strokes). The homogenate was diluted to 5 vol. with sucrose-tris-ME buffer and nuclei and intact cells were

removed by centrifuging at 750 g for 10 min. The mitochondria were then sedimented at 10,000 g for 10 min and washed twice by resuspension and centrifugation in sucrose-tris-ME buffer (one-half and one-fourth, respectively, of the original volume used for isolation). The washed mitochondrial pellet was resuspended in about 30ml of sucrose-tris-ME buffer. The exact volume was noted. Aliquots were taken for protein determination.

b) Preparation of mitochondrial inner membrane

Inner membrane was prepared by the digitonin procedure of Schnaitman and Greenawalt (1967, 1968) as described by See and Fitt (1971).

The washed mitochondria were collected by centrifugation at 10,000 g for 10 min and resuspended in sucrose-tris-ME buffer to give a suspension containing 100 mg protein/ml. Freshly prepared digitonin (1% w/v in sucrose-tris-ME buffer) was added to give a ratio of 1.1 mg digitonin/10 mg protein. The resulting solution was stirred gently for 15 min and then diluted with 3 vol. of sucrose-tris-ME buffer. The diluted suspension was homogenized (3 strokes) in a loose fitting Dounce homogenizer and centrifuged at 12,000 g for 15 min. The supernatant was discarded. The pellet was resuspended in the same volume of buffer, homogenized as before and centrifuged at 12,000 g for 10 min. The pellet obtained was resuspended in sucrose-tris-ME buffer (14ml) and was divided into two equal portions. Each sample was sonicated for 30 sec with a Bronwill Biosonik II sonic disintegrator (small probe; dial setting 20). They were then centrifuged for 30 min at 78,000 g (Beckman

type 30 rotor, 30,000 r.p.m.). The supernatant was discarded. The pellet was transferred to a Dounce homogenizer with a small volume of sucrose-tris-ME buffer and homogenized to a fine suspension. Sufficient sucrose-tris-ME buffer was added to give a final volume of 0.15ml/g wet weight liver. The inner membrane suspension was either stored at -20° or used immediately for the purification of polynucleotide phosphorylase (the enzyme is unstable once solubilized with Triton X-100). If the inner membranes were to be used directly for studies of the enzyme in the bound state, they were suspended in 0.2 to 0.25 ml sucrose-tris-ME buffer/gm wet weight liver.

c) Purification of mitochondrial polynucleotide phosphorylase

The frozen inner membrane suspension (see above) was thawed and diluted with an equal volume of cold (4°) 10% (v/v) Triton X-100-1mM-2-mercaptoethanol-0.01M-tris-HCl buffer, pH 7.4. The suspension was stirred for 20 min and then centrifuged for 30 min in a Beckman type 30 rotor at 30,000 r.p.m. The pellet was discarded and 10mg/ml Torula Yeast RNA in water was added to the supernatant to a final RNA concentration of 2mg/ml. Mn-RNA gel (1mg RNA/g wet wt. liver; for preparation, see below) was added and the suspension was stirred at 4° for 1 h. The gel was collected by centrifugation and the supernatant was treated as before with Mn-RNA gel. The combined Mn-RNA gel pellets were washed with sucrose-tris-ME buffer (10ml) by suspension in a Dounce homogenizer with loose-fitting pestle followed by centrifugation (only a small volume of buffer

should be used, since the Mn-RNA gel is fairly soluble). The washed pellet was resuspended in 10ml of 1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (tris-ME buffer) and applied at 60ml/h to a column, 1.7cm x 16cm, of DEAE cellulose (Whatman DE23) previously equilibrated with tris-ME buffer. When the Mn-RNA gel had settled on top of the column, it was overlaid with a layer of DEAE cellulose (about 3cm thick). The column was washed with tris-ME buffer until the eluate was free from material absorbing at 280nm. The enzyme was then eluted with 0.25M-NaCl in tris-ME buffer. The eluate was collected until no more protein emerged (total vol. 60-70ml) and 472g/ℓ (70% saturation) of solid $(NH_4)_2SO_4$ was added. The suspension was stirred for 20 min at 4° and the precipitate (not visible owing to large volume) collected by centrifuging at 16,000 g for 15 min. The very small yellow pellet was dissolved in tris-ME buffer (50μℓ/g wet wt. liver) to give a slightly cloudy, yellow solution which was dialysed overnight against tris-ME buffer (2ℓ). During the dialysis, the enzyme aggregated to give a fine white precipitate. The suspension was stored at 4° (freezing and thawing causes a 30-40% loss of enzymic activity at this stage).

Preparation of Mn-RNA gel. Mn-RNA gel was freshly prepared just before use. The preparation may be carried out at room temperature. The pH of an aqueous suspension of Torula yeast RNA was adjusted to 9 with ammonia and the mixture was stirred until all the RNA dissolved. The volume was adjusted with water to an RNA conc. of 10mg/ml. 1M-$MnCl_2$ (2 vol.)

was added and stirring was continued for a further 10 min. The Mn-RNA gel was collected by centrifugation and washed once with a volume of tris-ME buffer equal to that of the original RNA solution used and the gel was then suspended in the same volume of cold (4^{o}) buffer by means of a hand homogenizer. For use in the purification of the polynucleotide phosphorylase (see above), the suspension was assumed to contain 10mg of RNA/ml.

(ix) Isolation of ^{32}P-labelled rat liver mitochondria

Mitochondria were labelled with ^{32}P in their phospholipids by the procedure of McMurray and Dawson (1969) using the labelling period shown by these authors to give maximum incorporation into cardiolipin (inner membrane) and still maintained a high specific radioactivity in other phospholipids. ^{32}P-orthophosphate in dilute HCl (pH 2-3) was neutralized with 1M K_2HPO_4 to give a specific activity of 1mCi/mg Pi (final pH: 7.4). Male rats (average weight 340 gm) were injected intraperitoneally with the radioactive orthophosphate, under light ether anaesthesia: two rats received 2μCi of ^{32}P-Pi/gm body weight and one rat 1μCi/g body weight. The animals were fed except for the night before being sacrificed. They were decapitated 49 hours after receiving the injections, and their livers removed and the mitochondria isolated as described above (section viii) except that the removal of connective tissue with the tissue press, prior to homogenization, was not performed. The mitochondria were suspended in sucrose-tris-ME buffer (50ml) and their radioactivity determined by liquid scintillation

counting. 50µl samples of the suspension was added to 10ml of a toluene-based scintillation fluid containing 0.4% (w/v) PPO (2,5-diphenyloxazole) and 10% (v/v) Beckman Biosolve BBS-3. The radioactive mitochondria were used for the study of mitochondrial contamination in nuclei preparations.

(X) Isolation of mitochondria from other tissues and from liver of other animals.

Mitochondria from bovine liver, rooster liver, guinea pig liver, rabbit liver, gold fish muscle, rat heart, rat kidney, rat skeletal (thigh) muscle and rat spleen were isolated by the procedure described for rat liver (section viii). The final pellet of washed mitochondria was suspended in sucrose-tris-ME buffer (3-5 mg protein/ml).

(xi) Isolation of rat liver microsomes and soluble fraction

Rat liver was homogenized in sucrose-tris-ME buffer (5 vol.) in a motor-driven Potter-Elvehjem homogenizer. The nuclei and mitochondria were removed by centrifugation for 15 min at 12,000 g. The supernatant was then centrifuged at 30,000 r.p.m. in a Beckman type 30 rotor (78,000 g) for 90 min. The pellet (microsome) was suspended in sucrose-tris-ME buffer. The supernatant (soluble fraction) was collected.

(xii) Isolation of glycogen particles

Part of the supernatant remaining after the removal of mitochondria from a crude liver homogenate in sucrose-tris-ME buffer (section (viii)) was mixed with sufficient 2.2M sucrose-1mM-2-mercaptoethanol-10mM-tris HCl buffer, pH 7.4 to raise

the sucrose concentration to 0.44M. Two samples (6ml each) of this mixture were layered over 5ml 2.1M sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 and centrifuged at 40,000 r.p.m. (type 40 rotor, 105,000 g) for 1h. (Luck, 1961). The pellet (glycogen particles) was suspended in sucrose-tris-ME buffer (1ml).

(xiii) Isolation of rat liver plasma membrane

Rat liver plasma membranes were isolated from 15 g liver by the method of Neville (1960) as modified by Emmelot et al. (1964). A male rat (450 g) wasdecapitated and bled. The liver (15 g) was removed and chilled in 5ml of 0.25M sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl, pH 7.4 (sucrose-tris-ME buffer). The liver was weighed and dried with tissue paper. It was cut into small pieces with scissors and divided into 3 equal portions (5 g each). 5 g of liver was homogenized in 25ml of water containing 1mM $NaHCO_3$, pH 7.5 (prepared by adding 1M $NaHCO_3$ to distilled water to pH 7.5), with a loose fitting Dounce homogenizer (25 strokes). The homogenates (3 batches) were poured into 375ml of the same medium and stirred for 2 min. The diluted homogenate was filtered through 4 layers of cheese-cloth. The suspension was placed in 250ml centrifuge bottles and centrifuged at 1,500 g for 10 min. The supernatants were discarded. The pellet was resuspended in the same medium (5ml/bottle) and transferred to the Dounce homogenizer. Each bottle was rinsed with 2.5ml of medium and transferred to the homogenizer. The pellet was suspended by homogenization (3 strokes). 15 ml of medium were added and the suspension was cen-

trifuged at about 1,200 g in a clinical centrifuge, at 4°, for 10 min (rotor 213, International clinical centrifuge, model CL, dial setting 6). The centrifuge was decelerated slowly. The supernatant was carefully drawn off and discarded. The pellet was resuspended in 30ml of medium and homogenized (3 strokes) in the Dounce homogenizer and recentrifuged as before. The pellet was again resuspended in 10ml of medium and centrifuged as before. The final pellet was resuspended in 3.3ml (total volume) of medium. 3.1ml of this suspension was taken and 5.8ml of 69% (w/w) sucrose was added slowly to bring the density of the solution to 1.22. It was then subdivided into three equal portions and each portion was transferred to a SW25.1 centrifuge tube. Over each suspension was layered 45% (w/w) sucrose (8ml), 41% (w/w) sucrose (10ml), 37% (w/w) sucrose (4ml). Water was added carefully to fill the centrifuge tube to about 0.3cm from the top of the tube. Centrifugation was for 2 h. at 25,000 r.p.m. (63,000 g) in a Beckman L3-50 preparative ultracentrifuge. The plasma membrane was found as a well-defined layer between the 37% and 41% sucrose. The membrane material was collected with a Pasteur pipette and the resulting suspension was diluted with equal volume of 1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 and centrifuged in the clinical centrifuge (dial setting 6) for 30 min. The supernatant was carefully drawn off and discarded. The pellet (plasma membrane) was resuspended in sucrose-tris-ME buffer (0.5ml). The relative amounts of the various sucrose solutions used for preparing the gradient were originally suggested by Ray (1970).

(xiv) Isolation of rat liver nuclear membrane

Rat liver nuclei were isolated by a slightly enlarged scale of the Blobel and Potter procedure (Blobel and Potter, 1966; Whittle, Buschnell and Potter, 1968). One rat liver (15 gm) was homogenized in 2 volumes of 0.25M sucrose in TMK-ME buffer (50mM tris-HCl, pH 7.4, 25mM KCl, 5mM-$MgCl_2$, 1mM-2-mercaptoethanol) with a motor-driven potter Elvehjem homogenizer. The homogenate was filtered through four layers of cheese cloth and transferred to Beckman type 30 rotor polycarbonate tubes (5 ml each). 10ml of 2.3M sucrose in TMK-ME buffer was introduced with a syringe (no. 13 needle) into each tube and the solutions were mixed, to bring the sucrose concentration to 1.62M. The mixture was underlaid with 5ml of 2.3M sucrose in TMK-ME buffer and centrifuged at 30,000 r.p.m. (78,000 g) for 1 h. The supernatant containing cell debris was decanted and the side of the tube cleaned with absorbant paper. The white nuclear pellet was suspended in 2.3M sucrose in TMK-ME buffer (2 ml) and the sucrose concentration of the suspension was lowered gradually by the addition at 10 min intervals of four 0.5 ml and two 2 ml portions of TMK-ME buffer lacking sucrose. The nuclei were stirred gently after each addition of buffer.

Nuclear membrane was isolated by the method of Berezney, Funk and Crane (1970). The washed nuclei were suspended in 10ml of 0.25M sucrose in TMK-ME buffer (1.22 mg protein/ml) and digested with DNase I (50µg DNase/ml of solution) for 18 h, at 4^{o}. The membrane was centrifuged at 3,600 g for 15 min. and washed twice with 0.25M sucrose in TMK-ME buffer (10ml). The nuclear membranes were resuspended in 20ml of 0.25M sucrose in TMK-ME buffer. 1ml was removed and washed with 5ml of 0.25M sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl, pH 7.4 (sucrose-tris-ME buffer) and resuspended in sucrose-tris-ME buffer to give the washed crude nuclear membrane. To the remaining 19ml of membrane suspension, solid $MgCl_2$ was added to a final concentration of 0.5M. A large amount of protein was removed from the membranes by this treatment. The $MgCl_2$-treated membrane was collected by centrifugation at 27,000 g for 15 min, washed twice with sucrose-tris-ME buffer and resuspended in 1ml of this buffer. The supernatant from the $MgCl_2$ treatment was dialysed overnight against 2l of 1mM-2-mercaptoethanol-2mM-EDTA, pH 7 (ME-EDTA buffer). The precipitated protein was collected by centrifugation and resuspended in ME-EDTA buffer (2ml) to give the $MgCl_2$ soluble fraction. The washed crude membrane, the $MgCl_2$ treated membrane and the $MgCl_2$ soluble fraction were all assayed for polynucleotide phosphorylase activity.

(xv) Isolation of nuclei by a large scale modification of the Blobel and Potter procedure.

Six male rats (400-450 g) were used for this preparation. The liver (about 90 g) was homogenized in 2 vol. of 0.25M sucrose

in TMK-ME buffer as described in section (xiv). The homogenate was filtered through four layers of cheese-cloth and transferred to a precooled (4^{o}) 1ℓ measuring cylinder. 2 vol. of 2.3M sucrose in TMK-ME buffer was added and mixed by inverting the cylinder, to bring the sucrose concentration to 1.62M. Equal volumes (90 to 125ml) were transferred to 250ml plastic screw cap centrifuge bottles by means of measuring cylinder and were underlaid with 80ml of 2.3M sucrose in TMK-ME buffer. (The 2.3M sucrose was introduced to the bottom of the 1.62M sucrose layer through a glass tubing connected to a graduated separatory funnel. All solutions were kept in ice-bath but the transfer could be done at room temperature.) They were then centrifuged at 28,800 g (14,450 g average) in an International refrigerated centrifuge (872 rotor) for 5 h.

The white nuclear pellet was found at one corner on the bottom of the bottle, while a layer of cell debris extended across the top surface of the 2.3M sucrose layer and down the side of the bottle opposite the nuclei. The bottles were stood upright and the layer of cell debris was gently detached from the side with a spatula so that it floated to the surface. A small amount adhering to the side of the bottle could be wiped off later. The 1.62M sucrose layer and the floating cell debris were sucked off by means of a glass tube (Pasteur pipette broken at the neck of its constriction) connected to a water pump. In order to remove the cell debris efficiently, the suction was performed by leveling the opening of the glass tube on the surface of the liquid. In this way, most of the cell

debris was removed by the time the 2.3M sucrose layer was reached. The bottle was tilted towards the nuclear pellet and the remaining floating debris removed. When the liquid was just above the nuclear pellet, the bottle was tilted away from the pellet and the remaining liquid was quickly removed. The nuclear pellet would flow down and be last removed if this step was not done quickly. The cell debris adhering to the wall of the centrifuge bottle was wiped off with absorbent papers. Nuclei were also prepared by this procedure with sucrose solutions in 1mM-2-mercaptoethanol-1mM-tris-HCl buffer, pH 7.4 in place of TMK-ME buffer. The nuclei were used for the attempted extraction of polynucleotide phosphorylase as described in section (xviii).

(xvi) Isolation of nuclei by the 2.2M-sucrose procedure

The nuclei were isolated by the procedure of Chauveau et al. (1956) as described by See and Fitt (1970).

Six fed male rats (400-450 g) or guinea-pigs (500-600 g) were decapitated and bled. Their livers were removed and chilled in 100 ml of ice-cold 0.25M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (sucrose-tris-ME buffer). The tissue was weighed, dried with absorbent paper, transferred to 2.2M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (4ml/g wet weight liver) and cut into small pieces. The mixture was homogenized in a Lourdes model MM-1 homogenizer for 90 sec at full speed and the homogenate was filtered through four layers of cheesecloth. The filtrate was left at 0° for 20 min and was then centrifuged at 40,000 g for 1 h. The supernatants were discarded and

the walls of the centrifuge tubes were wiped carefully with absorbent paper. The red-coloured nuclei were used for extraction of polynucleotide phosphorylase.

(xvii) Isolation of nuclei by resuspension

Livers from six male rats (400-450 g) were homogenized and centrifuged as described for the isolation of mitochondria (section viii). The nuclear pellet was resuspended in 2.2M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (4ml/g wet weight liver) and homogenized in a Lourdes model MM-1 homogenizer for 90 sec at full speed. The nuclear suspension was left at 0^{o} for 20 min and was then centrifuged at 40,000 g for 1 h. The supernatant was discarded and the walls of the tube were wiped with absorbent paper. The reddish-white nuclear pellets were used for extraction of polynucleotide phosphorylase.

(xviii) Extraction and purification of polynucleotide phosphorylase from rat liver nuclei

The nuclei were transferred to a Dounce homogenizer with 0.25M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (sucrose-tris-ME buffer) (30ml) and homogenized (3 strokes) with a loose fitting pestle. The suspension was kept at 0^{o} for 20 min and then centrifuged at 40,000 g for 10 min. The supernatant was collected and the pellet was re-extracted twice with 30 ml portions of sucrose-tris-ME buffer. The supernatant from the three extractions were combined to give the crude sucrose extract. The pellet was discarded. The crude extract was dialyzed overnight against 2ℓ of 1mM-2-mercaptoethanol-2mM-EDTA buffer, pH 7.0.

The dialyzed sucrose extract was centrifuged at 40,000 g for 20 min to remove insoluble material. The pH of the stirred, cooled (0^{o}) supernatant was adjusted to 5.6 with 1M sodium acetate buffer, pH 5.3, and the suspension was stirred for 1 min. The precipitate was collected by centrifugation and dissolved in 20ml of 2mM-EDTA-1mM-2-mercaptoethanol-10mM-tris-HCl, pH 7.4 (tris-ME-EDTA buffer). The cloudy solution was centrifuged and the pellet was discarded. The supernatant was applied, the same day, (at 30ml/h) to a column (1.7cm x 16cm) of DEAE-cellulose (Whatman DE23) previously equilibrated with tris-ME-EDTA buffer. The column was washed with tris-ME-EDTA buffer until no more protein emerged, and the enzyme was then eluted at 50ml/h with 0.3M-NaCl in tris-ME-EDTA buffer (30ml; 3ml fractions). The individual fractions were dialysed together overnight against 1mM-2-mercaptoethanol-2mM EDTA buffer, pH 7.0 (2 h) and assayed for enzymic activity. All the active enzyme was precipitated during the dialysis. The active precipitate were collected by centrifugation and washed twice by resuspension in the dialysis buffer (3ml) followed by centrifugation. The solid was collected and stirred with dialysis buffer (3ml) until a fine suspension was obtained.

(xix) Extraction and purification of guinea-pig nuclear polynucleotide phosphorylase

The nuclei prepared by the 2.2M sucrose procedure (xvi) were extracted (3 times) with 0.25M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (sucrose-tris-ME buffer) as described for extraction of rat liver nuclei (xviii) to give the crude

sucrose extract . The nuclear pellet was re-extracted twice with 30ml portions of sucrose-tris-ME buffer, and the supernatants were discarded. The pellet was then extracted three times, the same procedure being used, with 0.14M-NaCl-1mM-2-mercaptoethanol-2-mM-EDTA, pH 7.0 to give the crude saline extract. Both the crude sucrose and saline extracts were dialysed separately overnight against 2ℓ of 1mM-2-mercaptoethanol-2mM-EDTA buffer, pH 7.0 (ME-EDTA buffer).

The dialysed sucrose extract was centrifuged at 40,000 g for 20 min to remove insoluble material. The pH of the stirred, cooled (0^{o}) supernatant was adjusted to 5.7 with 1M-sodium acetate buffer, pH 5.3, and the suspension was stirred for 1 min. The precipitate was collected by centrifugation and dissolved in 40 ml of 2-mM-EDTA-1M-2-mercaptoethanol-10mM-imidazole-HCl buffer, pH 7.2 (imidazole-ME-EDTA buffer). The cloudy solution was centrifuged and the pellet discarded. The enzyme was purified by DEAE cellulose column chromatography as described for the rat liver nuclear enzyme (xviii) with imidazole-ME-EDTA buffer in place of tris-ME-EDTA buffer. The final purified particulate enzyme was resuspended in 5ml of ME-EDTA buffer and referred to as "sucrose fraction".

A purer particulate enzyme (specific activity about 1100-1200 units/mg) could be obtained if the DEAE-cellulose column was eluted stepwise with 0.1M-, 0.2M- and 0.3M-NaCl in imidazole-ME-EDTA buffer. However, the yield was greatly decreased. The simplified procedure described above was used in routine work, since relatively large amounts of enzyme were needed and the only significant difference between the products obtained by the two

methods appeared to be a slightly greater contamination of the less pure enzyme by phosphatases.

The dialysed saline extract was centrifuged at 40,000 g for 20 min to remove solid material. The pH of the clear supernatant was adjusted to 5.7 with 1M-sodium acetate buffer, pH 5.3, and the mixture was stirred for 1 min. The precipitate was collected by centrifugation, suspended in ME-EDTA buffer (1 ml) and dialysed overnight against ME-EDTA buffer (200 ml). This particulate enzyme fraction is referred to as the "saline fraction".

(x) Isolation of rat liver mitochondrial polynucleotide phosphorylase by osmotic shock

Six male rats (400-450 g) were used for this experiment. Mitochondria were isolated as described in method (viii). The mitochondria were subdivided into two equal portions. To each mitochondrial pellet, 2.2M sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl, pH 7.4 (5ml) was added and stirred with glass rod. Each suspension was transferred to a small Potter-Elvehjem homogenizer (10ml capacity) and homogenized gently, by hand (3 strokes). The suspensions were allowed to stand at 0^{o} for 20 min and 1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (tris-ME buffer) were added to bring the sucrose concentration to 0.25M. These suspensions were homogenized in a loose fitting Dounce homogenizer (3 strokes) and allowed to stand for 20 min. The diluted suspensions were centrifuged at 40,000 g for 10 min. The supernatants were collected and the pellets were further treated as described above. The two supernatants were pooled and dialyzed overnight

against 1mM-2-mercaptoethanol-2mM-EDTA, pH 7.0 (ME-EDTA buffer). The dialysed solution was centrifuged at 40,000 g for 10 min and the pellet discarded. The supernatant was treated as described for the nuclear extracts (method xviii) to isolate polynucleotide phosphorylase. The solid (enzyme) obtained was resuspended in ME-EDTA buffer (0.5 ml).

II. C. Results

1. Rat liver mitochondrial polynucleotide phosphorylase

(i) Product specificity of rat liver mitochondrial polynucleotide phosphorylase

Rat liver mitochondrial inner membranes were prepared by the digitonin procedure as described in the Methods. The standard phosphorolysis assay was scaled up three-fold, with either (a) poly C or (b) poly A as substrate. The reaction mixtures were incubated at 37°, in a water bath-shaker, for 2 h in the presence of mitochondrial inner membranes (0.47 mg protein). Controls without enzyme were run in each case. The reaction was stopped by the addition of charcoal/$HClO_4$ 1% (w/v) charcoal in 2.3% (w/v) $HClO_4$ (1 ml). The mixture was kept at 0° for 10 min, and 1.25mM-KH_2PO_4 in 1% (w/v) $HClO_4$ (2 ml) then added. The charcoal was collected and washed five times with 1.25mM-KH_2PO_4 in 1% (w/v) $HClO_4$ (3 ml) and three times with water (3 ml). It was transferred with water (1 ml) to a Pasteur pipette fitted with a cotton-wool plug covered with cellulose powder. The nucleotides were eluted with 2% (v/v) NH_3 in 50% (v/v) ethanol (2 ml). The eluate was lyophilised. The nucleotides were redissolved in water (50µl) and applied to a thin layer chromatographic plate coated with Serva TLC-DEAE-cellulose. The chromatogram was developed with 4% (w/v) formic acid (Fahn, Albers and Koval, 1965). The nucleotides were located with ultra-violet light and the area corresponding to each of the standard nucleotides (NTP,

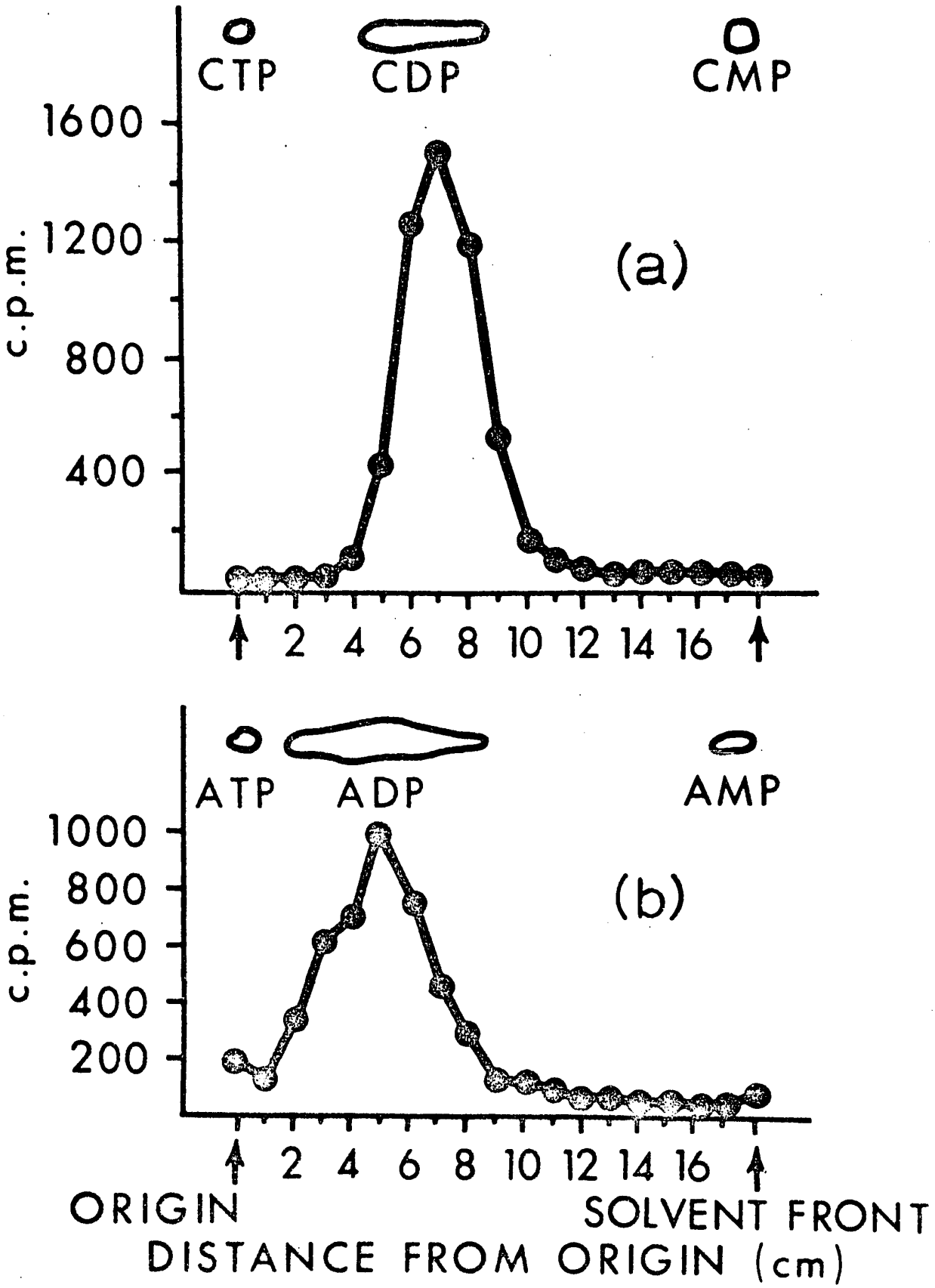


Figure 1: Product specificity of rat liver mitochondrial polynucleotide phosphorylase.

NDP and NMP) was noted. Successive areas (1 cm long) of DEAE-cellulose were then scraped from the plates and added to 5 ml of toluene based scintillation fluid containing 0.5% (w/v) PPO(2,5-diphenyloxazole) and 2% (v/v) ethanol. The radioactivity was determined in a Beckman LS-133 liquid scintillation counter.

The products obtained from the phosphorolysis of poly C and poly A were almost exclusively ^{32}P-labelled CDP and ADP respectively (Fig. 1). In the case of the phosphorolysis of poly A, a small amount of ^{32}P-labelled ATP was obtained (Fig. 1b). This is due to the presence of adenylate kinase in the inner membrane preparation. Table 1a shows that when ^{14}C-ADP was incubated with the inner membrane, about 9% of the ADP was converted to ATP by adenylate kinase which catalysed the reaction.

$$2ADP \rightleftharpoons ATP + AMP$$

If adenylate kinase is the only enzyme present, the amount of AMP should be the same as the amount of ATP produced. However, the amount of AMP produced is much greater than ATP and, this means that the inner membrane contains phosphatase(s) that degrade ADP to AMP. Table 1b shows that there was little or no cytidylate kinase activity present in the inner membrane. The inner membrane contains a phosphatase that degrades CDP to CMP. Rat liver mitochondrial inner membrane preparations have been shown to contain 0.2% of the total mitochondrial adenylate kinase (Schnaitman and Pederson, 1968) and 10% in the case of beef heart mitochondria (Kopaczyk et al., 1968).

When ATP or AMP (1mM) replaced poly A or poly C in the conditions described above for the phosphorolysis of poly A or poly C,

TABLE 1

Adenylate kinase and cytidylate kinase activity of rat liver mitochondrial inner membrane preparation

The adenylate kinase was determined with 1mM ^{14}C-ADP and 574 µg inner membrane protein as described in the Methods section. Cytidylate kinase activity was determined as for adenylate kinase with ^{14}C-CDP replacing ^{14}C-ADP.

(a) adenylate kinase

Products	radioactivity (c.p.m.)	Percent of total radioactivity
ATP	3517	8.7
ADP	11526	28.6
AMP	25182	62.7

(b) cytidylate kinase

CTP	45	0.3
CDP	11561	80.3
CMP	2797	19.4

no ^{32}P-labelled nucleotides could be detected. These results discounted the possibility that the ^{32}P-labelled ADP obtained in Fig. 1 was formed by the following reactions:

(a) $ATP \longrightarrow ADP + P_i$

(b) $ATP + AMP \longrightarrow ADP$

(c) $AMP + P_i \longrightarrow ADP$

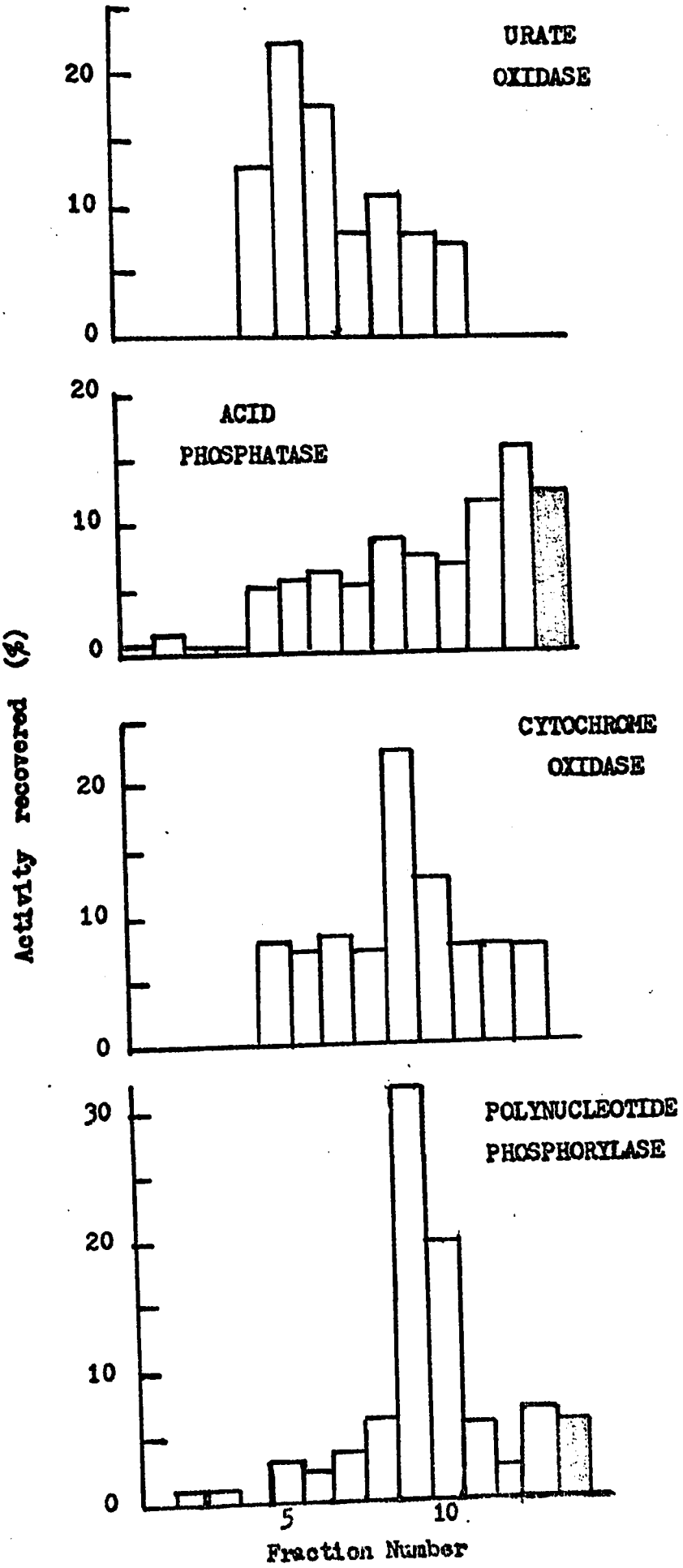
These results therefore demonstrated that the mitochondrial inner membrane preparation contains polynucleotide phosphorylase.

(ii) Distribution of polynucleotide phosphorylase activity after isopycnic centrifugation of rat liver mitochondrial preparation

Rat liver mitochondrial preparations contain mitochondria, peroxisomes and lysosomes (Beaufay et al., 1964). In order to show that polynucleotide phosphorylase is present in mitochondria and not due to contamination by peroxisomes or lysosomes, the three particles were separated by isopycnic centrifugation, of the mitochondrial preparation, in sucrose-glycogen gradient (see Methods).

The distribution of the marker enzymes, urate oxidase (peroxisomes), acid phosphatase (lysosomes) and cytochrome oxidase (mitochondria), is shown in Fig. 2 and is very similar to that reported by Beaufay et al., (1964). The bulk of the urate oxidase (peroxisomes) accumulated at the upper regions of the gradient and the acid phosphatase (lysosomes), although dispersed throughout the gradient, is concentrated in the lower regions and in the sediment. Cytochrome oxidase (mitochondria) occupies

Figure 2: Distribution of polynucleotide phosphorylase activity after isopycnic centrifugation of rat liver mitochondrial preparation. Sucrose-glycogen gradient centrifugation was carried out as described in Methods. The marker enzymes for peroxisome (urate oxidase), mitochondria (cytochrome oxidase) and lysosomes (acid phosphatase) (Beaufay, et al., 1964) were assayed as described in Methods. Polynucleotide phosphorylase was determined by the standard poly A phosphorolysis assay, but the time of incubation was 3 hours. All assays were performed in the presence of 0.1% (v/v) Triton X-100. The shaded areas represent the pellets at the bottom of the tubes.


URATE
OXIDASE
ACID
PHOSPHATASE
CYTOCHROME
OXIDASE
POLYNUCLEOTIDE
PHOSPHORYLASE
Activity recovered (%)
0
10
20
30
5
10
Fraction Number

an intermediary positions. Polynucleotide phosphorylase have the same distribution as cytochrome oxidase and is therefore present in the mitochondria and not in lysosomes or peroxisomes. Since most of the lysosomal enzymes have maximum activity in the acid pH range (pH optimum at about 5) (De Duve, 1963; Gahan, 1967), polynucleotide phosphorylase activity was assayed at pH 5.1 in acetate buffer. There was no detectable activity at this pH and thus, confirmed the conclusion drawn above that polynucleotide phosphorylase in the crude mitochondrial preparation is not from lysosomal contamination.

The standard phosphorolysis assay used does not distinguish between ^{32}P-labelled nucleotides produced by phosphorolysis of poly A (ADP) or by oxidative phosphorylation (ATP). In order to show that the labelled nucleotides were produced by phosphorolysis, the enzymic activity was assayed in the absence of poly A. There was no significant formation of charcoal adsorbable radioactivity (Table 2). It should be emphasized that the standard phosphorolysis assay used (for mitochondrial enzyme) contains 0.1% (v/v) Triton X-100 which solubilized (visually) the mitochondria used in the assay; and in this condition (in the absence of vesicle) oxidative phosphorylation does not take place (Racker, 1970).

(iii) Submitochondrial localization of rat liver mitochondrial polynucleotide phosphorylase

Rat liver mitochondria were subfractionated as described in Methods. Mitochondria were separated into outer membrane and

TABLE 2

Polynucleotide phosphorylase activity assayed in the presence and absence of poly A

Polynucleotide phosphorylase was assayed in the standard poly A phosphorolysis assay in the presence and absence of poly A.

Experiment	$^{32}P\text{-}P_i$ incorporated (c.p.m.)
+ poly A, - mitochondria	145
+ poly A, + mitochondria	2155
- poly A, + mitochondria	169

TABLE 3

Intra-mitochondrial distribution of polynucleotide phosphorylase

Mitochondria were fractionated by digitonin treatment and differential centrifugation and enzymic activities were assayed on each fraction as described in the Method section.

Fractions	Marker Enzymes		
	NADH-cyt. c reductase (rotenone insensitive)	Succinate-cyt. c reductase	Polynucleotide phosphorylase
	µmoles cyt. c reduced/min/mg protein		nmoles ^{32}P-P_i incorp./hr/mg protein
whole mitochondria	0.30	0.063	119
inner membrane-matrix fraction	0.17	0.144	210
broken mitochondria	0.44	0.022	10
outer membrane	1.46	0.0004	0
inter-membrane space	0.14	0.000	15

TABLE 4

Polynucleotide Phosphorylase Activity

in Sub-Mitochondrial Fractions

Rat liver mitochondria were fractionated as described in Methods. Sonication of the inner membrane-matrix fraction followed by centrifugation at 78,000 g for 1.5 h gave the matrix (supernatant) and inner membrane (pellet) fractions. Polynucleotide phosphorylase activity was determined by the standard assay (Methods).

Fraction	Total Units (nmoles ^{32}P-P_i incorporated/hr)	Recovery (%)
Whole mitochondria	13902	100
inner membrane-matrix fraction	12640	91
broken mitochondria	112	1
outer membrane	0	0
inter-membrane space	225	2
Fractionation of inner membrane-matrix fraction:		
inner membrane (pellet)	12375	89
matrix (supernatant)	135	1

inner membrane-matrix fractions by digitonin treatment followed by differential centrifugation (Schnaitman and Greenawalt, 1967, 1968). Rotenone-insensitive NADH-cytochrome c reductase and succinate-cytochrome c reductase were used as marker enzymes for the outer and inner membranes, respectively (Schnaitman and Greenawalt, 1968; Sottocasa, et al., 1967). Table 3 shows the intra-mitochondrial distribution of polynucleotide phosphorylase: it can be seen that its specific activity was highest in the inner membrane-matrix fraction. Table 4 shows that 89% of the total polynucleotide phosphorylase activity of the crude extracts (corresponding to almost 100% of the activity in the inner membrane-matrix fraction) was recovered in the inner membrane pellet. This final fraction also contained 86% of the total succinate-cytochrome c reductase activity. These results show that rat liver mitochondrial polynucleotide phosphorylase is located in the inner membrane of the organelle.

(iv) Purification and properties of rat liver mitochondrial polynucleotide phosphorylase

The method of purification of rat liver mitochondrial polynucleotide phosphorylase described in the Methods section has been repeated six times and the results of the three most recent purifications, in which the fate of the ADP/P_i-exchange activity was followed as well as that of the phosphorolytic activity, are shown in Table 5.

The partially purified enzyme was obtained in particulate form: the 0.25 M-NaCl fraction from the DEAE-cellulose chromatography step was clear and the solution after subsequent

TABLE 5

Purification of rat liver mitochondrial polynucleotide phosphorylase

The enzyme was purified as described in the Methods section and poly A phosphorolysis and ADP/P_i exchange activities were determined by their respective standard assays.

Prepn.	Fraction	Phosphorolysis activity		Exchange activity	
		units	units/mg protein	units	units/mg protein
1	whole mitochondria	54120	191	91896	324
	inner membrane	44776	276	70222	431
	purified enzyme	24539	1624	4400	291
2	whole mitochondria	30203	120	50641	201
	inner membrane	28501	182	46592	297
	purified enzyme	18385	1332	8233	581
3	whole mitochondria	-	-	-	-
	inner membrane	38931	113	82624	240
	purified enzyme	10008	1713	0	0

TABLE 6

Effect of removal of lipids on mitochondrial polynucleotide phosphorylase activity

Mitochondria were isolated as described by Method viii from 3 rat livers. The washed mitochondrial suspension was divided into two equal fractions and the mitochondria were collected by centrifugation at 10,000 g for 10 min. Each pellet was resuspended in 2.5 ml of isolating buffer. The lipids were removed by treatment with 96% (v/v) acetone (to remove neutral lipids) or 90% (v/v) acetone (to remove phospholipids and neutral lipids) exactly as described by Lester (1967). The lipid depleted mitochondria were suspended in 5 ml of 0.88M sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl, pH 7.4 and assayed for polynucleotide phosphorylase activity.

Experiments	Total units	Percent
untreated	2064	100
96% acetone treatment	1990	96
90% acetone treatment	1620	79

$(NH_4)_2SO_4$ concentration was only slightly cloudy, but large particles formed during the final dialysis against the low ionic strength tris-ME buffer. Addition of Triton X-100 to the 0.25M NaCl used for elution of the enzyme from the column had no effect on the recovery or properties of the final product. The enzyme could not be sedimented by centrifugation at 78,000 g for 30 min of the initial Triton X-100 extract of the mitochondrial membranes, nor did it become particulate during dialysis of the extract against tris-ME buffer, so it appears that aggregation of the enzyme is favoured by preliminary exposure to 0.25 M-NaCl followed by dialysis against the buffer of low ionic strength.

The partially purified, particulate mitochondrial enzyme usually contained small amounts of phospholipids, mainly cardiolipin and phosphatidylethanolamine, but the total and relative amounts of the latter varied from one preparation to another and they were undetectable on one occasion. Therefore, the polynucleotide phosphorylase does not require lipid for activity although it is membrane-associated. This conclusion is supported by the observation that extraction of intact mitochondria with either 96% (v/v) acetone, which removed all of the neutral lipid (Lester, 1967) or 90% (v/v) acetone, which removed 80% of total phospholipids (Lester, 1967), did not affect the phosphorolytic activity greatly (Table 6). These treatments are known to inactivate enzymes of the electron-transport chain (Fleischer and Fleischer, 1967), which can then be reactivated by recombination with the lipids. The acetone treatment however, rendered mitochondrial polynucleotide phosphorylase insoluble in Triton X-100.

The final specific phosphorolytic activity of the partially purified enzyme was remarkably constant, with a maximum variation of less than 30%, but the recovery and purification of the associated ADP/P_i exchange activity varied markedly. The more purified the enzyme, in terms of phosphorolytic activity, the lower the exchange activity detected and, in one case, the preparation was completely free of the exchange activity (table 5). These observations support the conclusion (see below) that the polynucleotide phosphorylase and exchange activities are due to different enzymes.

During storage at 4^{o} , the partially purified preparation was stable overnight and lost 44% of its phosphorolytic activity and 53% of its exchange activity in one week. However, if the enzyme was frozen and thawed after overnight storage, it lost 30-40% of its phosphorolytic activity.

It is possible to obtain a soluble polynucleotide phosphorylase preparation by the following procedure. The polynucleotide phosphorylase was solubilized by treating the mitochondrial inner membrane obtained from 3 rat livers with 0.1% (v/v) Triton X-100 (3 ml/g wet wt. liver) and precipitated with $(NH_4)_2SO_4$ (40-80% saturation). The precipitate was dissolved in 1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (tris-ME buffer) (1 ml) and dialysed overnight, against tris-ME buffer, to remove $(NH_4)_2SO_4$. The yellow solution obtained contained about 50% of the initial solubilized polynucleotide phosphorylase activity. No activities could be recovered in the 0-40% or 80-90% $(NH_4)_2SO_4$ fractions. This soluble form of enzyme was very unstable,

losing 50-60% of its activity during overnight storage at 4° or -20°, and all its activity at room temperature during the same period. The enzyme could not be stabilized by varying the pH of the buffer medium over the range 7.2-9.2. Lipids, phosphatases and some adenylate kinase were present in these preparations. The phosphatase(s) could not be separated from the polynucleotide phosphorylase by column chromatography on DEAE-cellulose or by acetone fractionations. Attempts to purify the polynucleotide phosphorylase further by Sephadex G-200 gel filtration, in the presence or absence of Triton X-100, led to complete loss of activity. Treatment of the initial inner membrane suspension with sodium cholate to remove adenylate kinase (Kopaczk et al., 1968) resulted in complete inactivation of polynucleotide phosphorylase activity.

The properties of the polynucleotide phosphorylase are unaltered by removal from the mitochondrial inner-membrane except for a change in its pH optimum. In the membrane-bound state, the latter was 7.8 although a plateau extending to pH 8.2 was observed with some preparations. In contrast (fig. 3), the partially purified enzyme had an invariant pH optimum at 8.2. This difference was probably due to the strong phosphatase activity in the inner membrane preparations (see p.172), which had a pH optimum of 8 and was entirely eliminated during the RNA-gel/DEAE cellulose step of the purification procedure.

The enzyme required Mg^{2+} for activity (fig. 4), with an optimum concentration of 6mM. Mn^{2+} could partially replace Mg^{2+}, but no phosphorolysis occurred in the presence of Ca^{2+}.

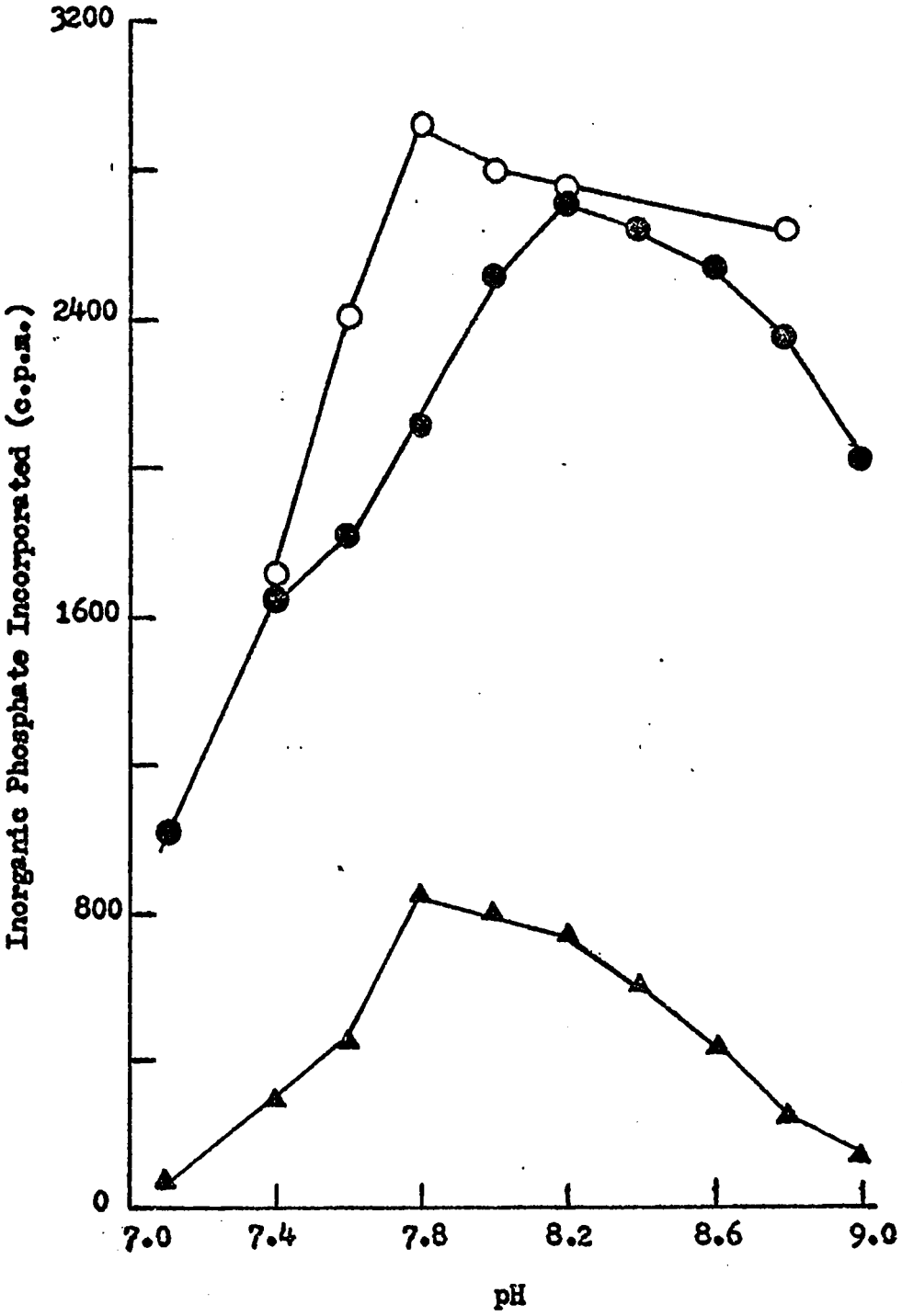


Figure 3: Dependence of phosphorolysis and exchange activities on pH. The polyA phosphorolysis and ADP/Pi exchange activities were measured in the standard assay conditions (Methods section) with buffers (10 mM Tris-HCl) of the indicated pH. phosphorolysis, purified enzyme,(●); phosphorolysis, crude inner membrane,(○); exchange, purified enzyme (▲).

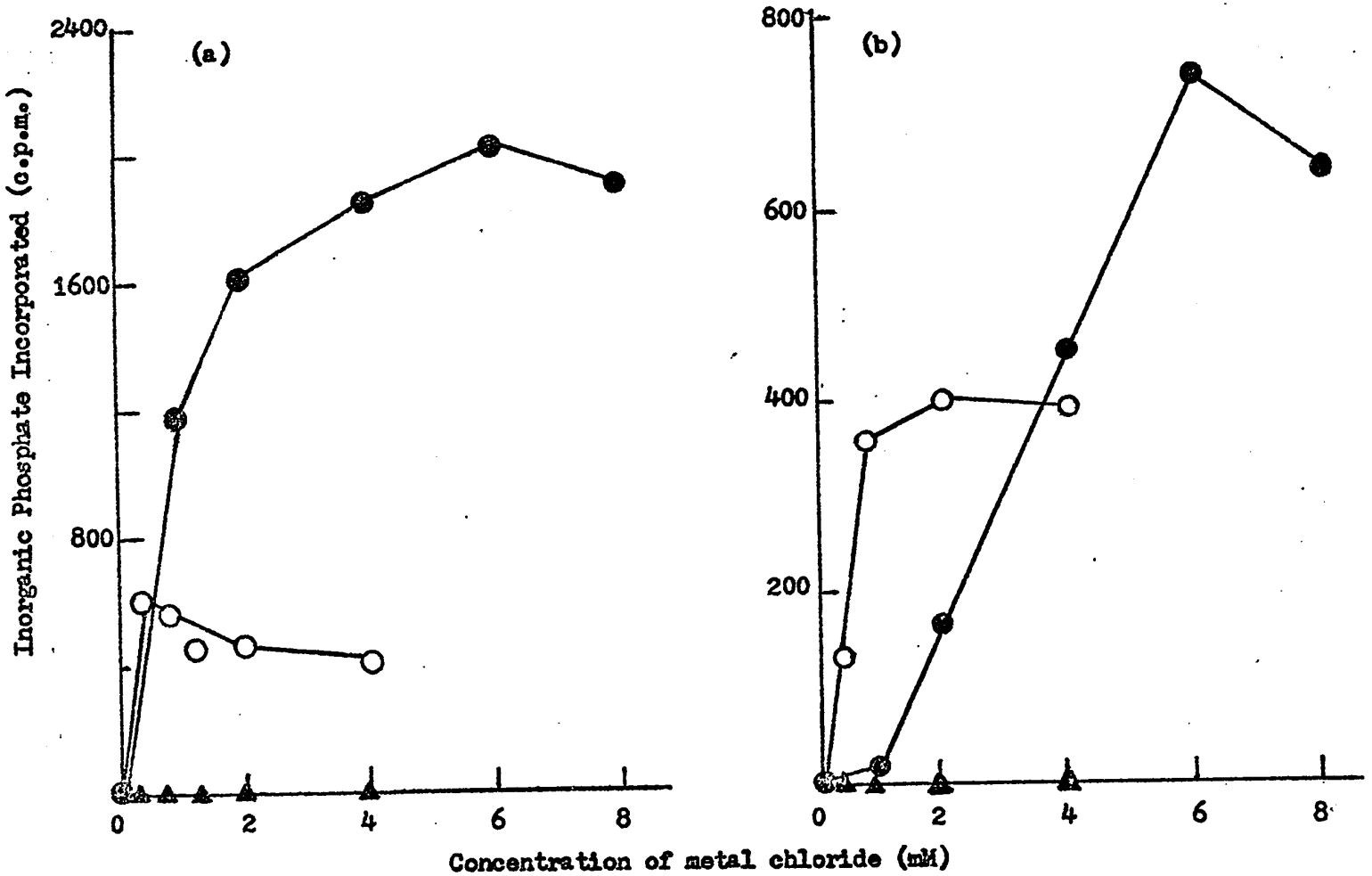


Figure 4: Influence of divalent cations on the phosphorolysis and exchange activities of rat liver mitochondrial polynucleotide phosphorylase. The partially purified enzyme was assayed (a) for polyA phosphorolysis activity and (b) ADP/Pi exchange activity by the appropriate standard assay (Methods section) with the indicated concentrations of $MgCl_2$ (●), $MnCl_2$ (○) and $CaCl_2$ (▲).

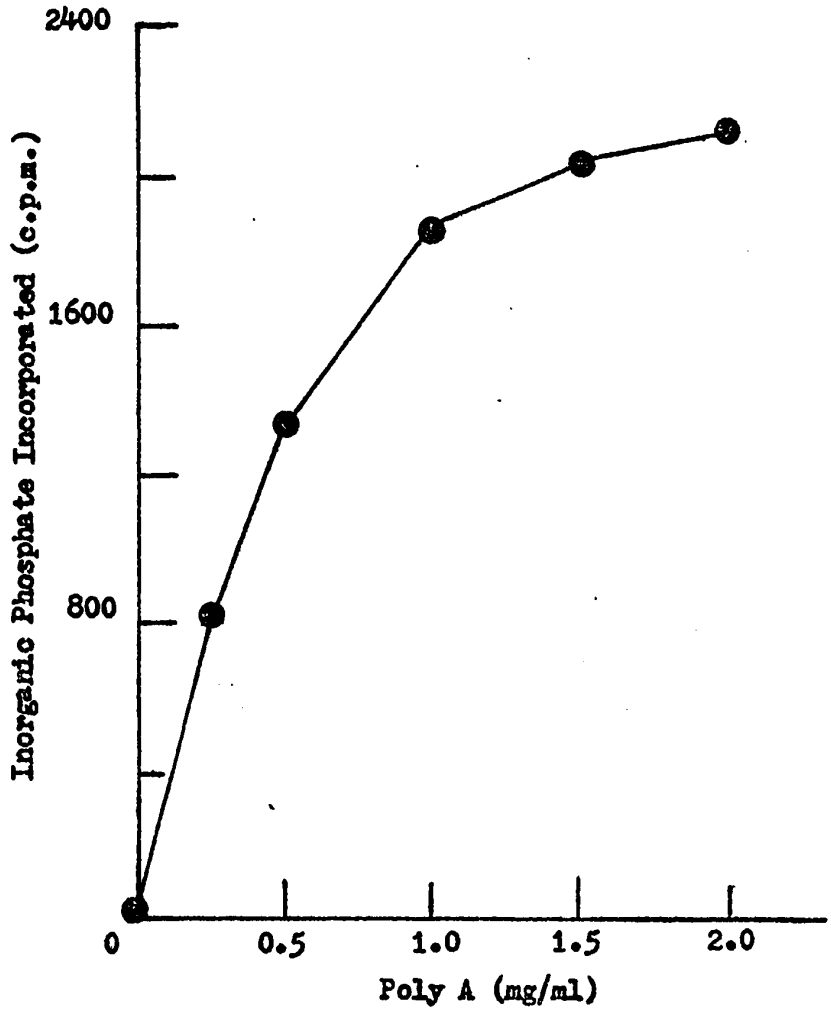


Figure 5: Substrate-saturation curve for rat liver mitochondrial polynucleotide phosphorylase. The phosphorolysis activity was measured in the standard assay with the indicated concentration of poly A.

TABLE 7

Substrate specificity of the phosphorolysis activity of mitochondrial polynucleotide phosphorylase

Phosphorolysis activity was measured by the standard assay at pH 8.2 with the partially purified enzyme and at pH 7.8 with the inner membrane suspension (the optimum conditions for each fraction). Controls (i) without enzyme and (ii) with enzyme but without substrate were essentially identical and their values (approximately 100 c.p.m.) were subtracted from the appropriate values. The enzyme fractions were from prepn. no. 1 (Table 5).

substrate	^{32}P-P_i incorporated (c.p.m.)	
	inner membranes	purified enzyme
poly A	2274	2013
poly C	2481	1379
poly G	2	11
poly U	1722	1139
wheat germ RNA	349	230
calf thymus DNA (native or heat denatured)	0	0

TABLE 8

Hydrolysis of nucleoside diphosphates by rat liver mitochondrial inner membrane phosphatase

Phosphatase activity of the mitochondrial inner membrane was measured as described in Section III B, using 0.5 mg membrane protein.

Substrate	μmole P_i released/30 min
ADP	0.40
CDP	0.09
GDP	0.18
UDP	0.20

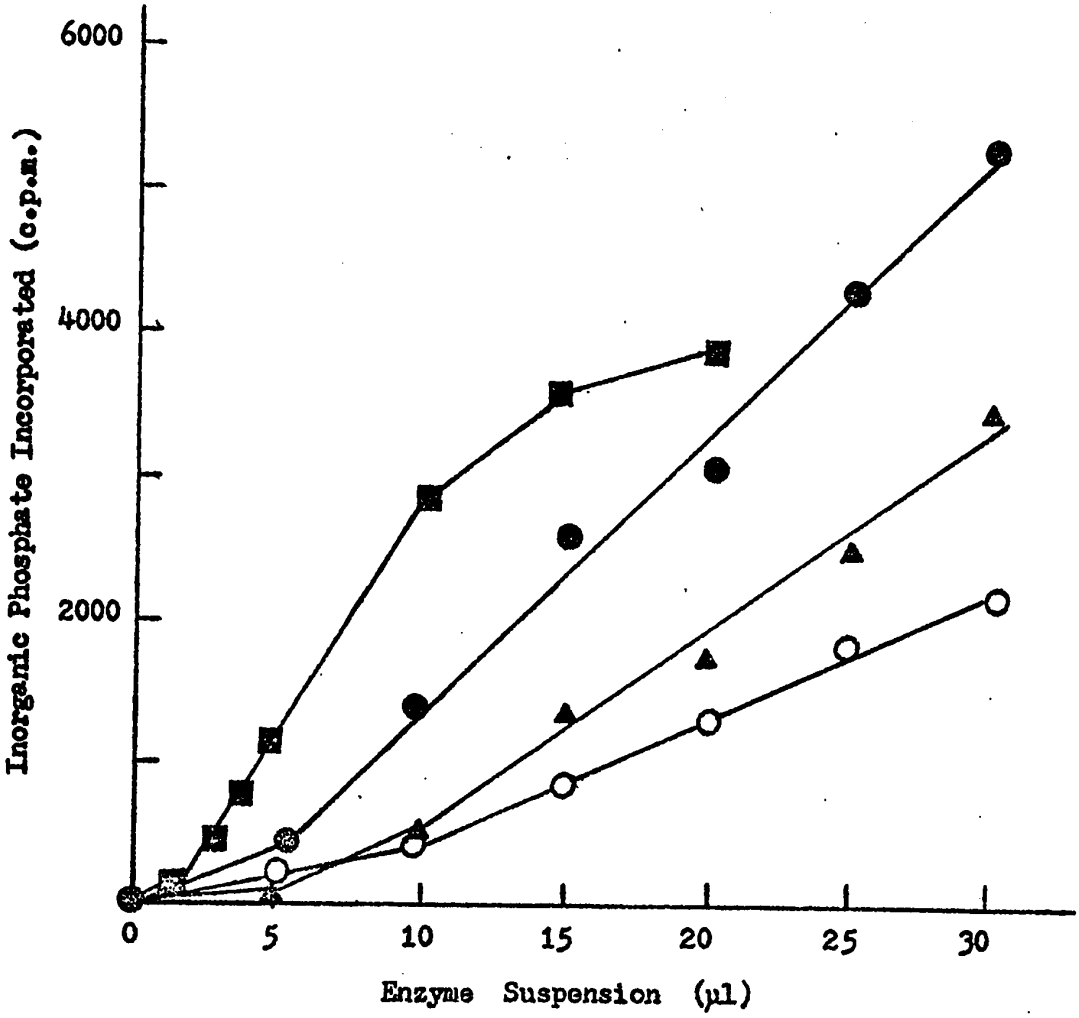


Figure 6: Enzyme-saturation curve for rat liver mitochondrial polynucleotide phosphorylase in the presence and absence of Triton X-100. The phosphorolysis activities of the crude mitochondrial inner membrane and the partially purified enzyme were measured in the standard assay conditions with and without 0.1% (v/v) Triton X-100 as indicated. Partially purified enzyme (2.9 mg protein/ml), with Triton X-100,(■); inner membrane suspension (5.87 mg protein/ml), with Triton X-100,(●); inner membrane suspension (1:2 dilution), with Triton X-100, (○); inner membrane suspension (5.87 mg protein/ml) without Triton X-100,(▲).

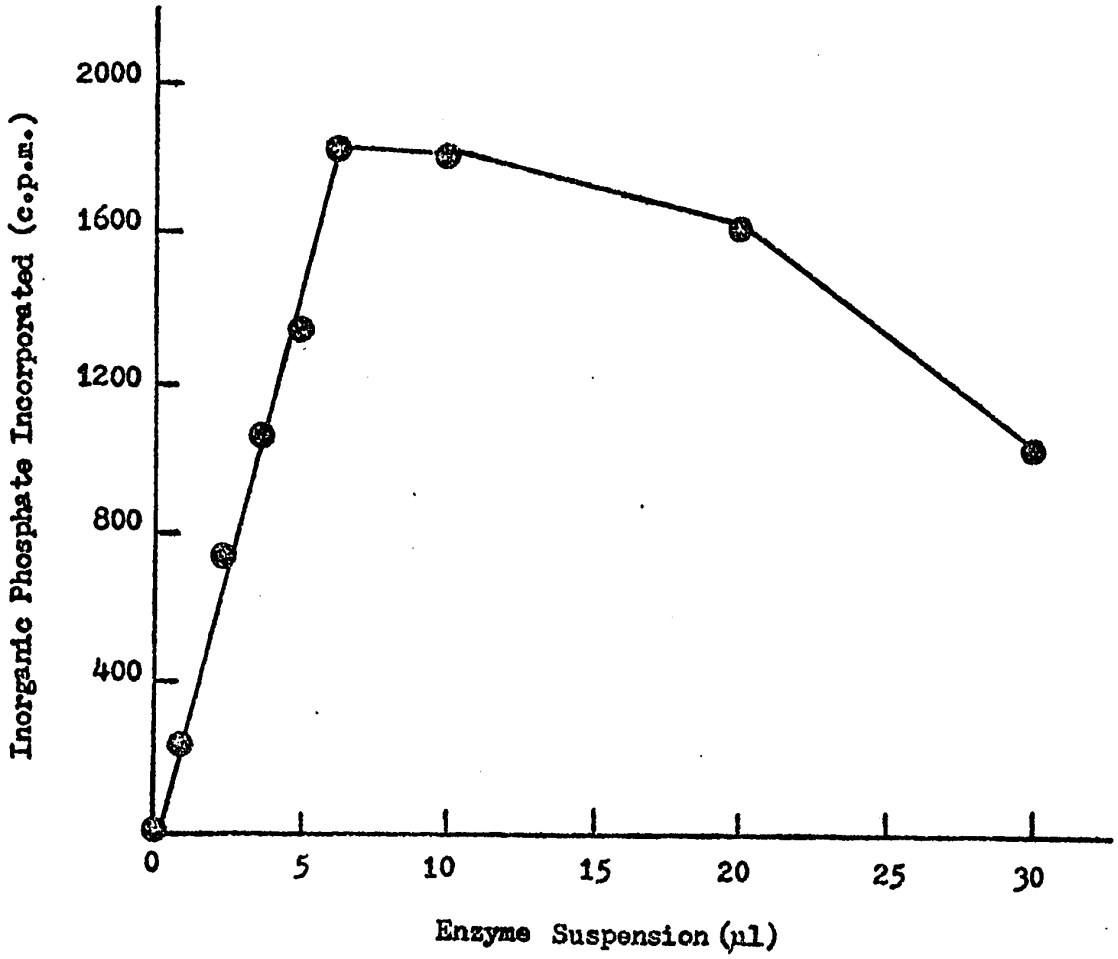


Figure 7: Enzyme-saturation curve for rat liver mitochondrial inner membrane polynucleotide phosphorylase. Poly A phosphorolysis in the standard phosphorolysis conditions with the indicated amount of inner membrane suspension (26.21 mg protein/ml).

The poly A saturation curve (Fig. 5) of partially purified mitochondrial polynucleotide phosphorylase reached a plateau at 1 mg/ml. Table 7 shows the specificity of both the purified and membrane-bound enzyme with a variety of polynucleotides, all used at the saturating concentration for poly A. Both the crude and partially-purified enzymes phosphorolysed poly A, poly C and poly U efficiently and high-mol. wt. wheat germ RNA to some extent. Little or no reaction occurred with poly G and DNA was not a substrate (Table 7). These results are in general agreement with those previously reported for bacterial polynucleotide phosphorylases (Grunberg-Manago, 1963).

Poly A was a significantly better substrate for the partially purified enzyme than either poly C or poly U, but the inner membrane fraction was slightly more active with poly C (Table 7). This apparent difference was due to the phosphatase(s) that are removed during the purification and which are much less active with CDP than with either ADP or UDP (table 8). The properties and substrate specificity of the inner-membrane phosphatase(s) will be described in more detail later (p. 172).

Figure 6 shows enzyme saturation curves for the crude inner membrane fraction and the partially-purified enzyme. In the standard assay conditions, the rate of phosphorolysis of poly A was proportional to enzyme concentration in the presence of 5-40 units of the crude preparation or 1-50 units of the purified polynucleotide phosphorylase. The crude inner membrane suspension gave a bell-shaped saturation curve (Fig. 7), so that the measured activity fell above 40 units/0.1 ml (about 200 µg protein/0.1 ml), but

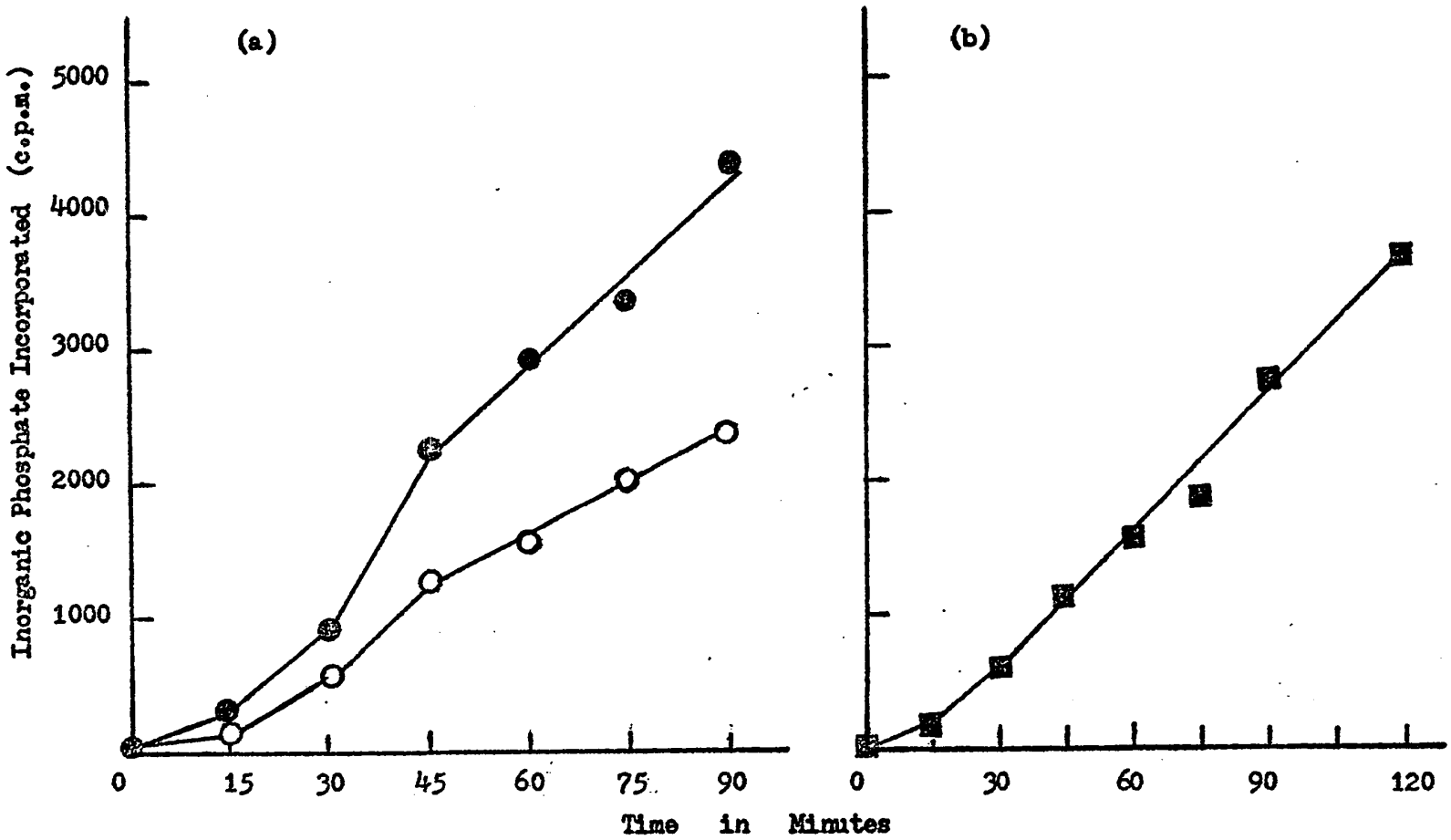


Figure 8: Time-course of poly A phosphorolysis by rat liver mitochondrial polynucleotide phosphorylase. Poly A phosphorolysis was measured in the standard assay condition. The reaction mixtures (without $MgCl_2$) were preincubated for 5 minutes at 37° C and at zero time, the reactions were started by the addition of $MgCl_2$. (a) inner membrane with (●) and without (○) Triton X-100 and (b) purified enzyme with Triton X-100 (■).

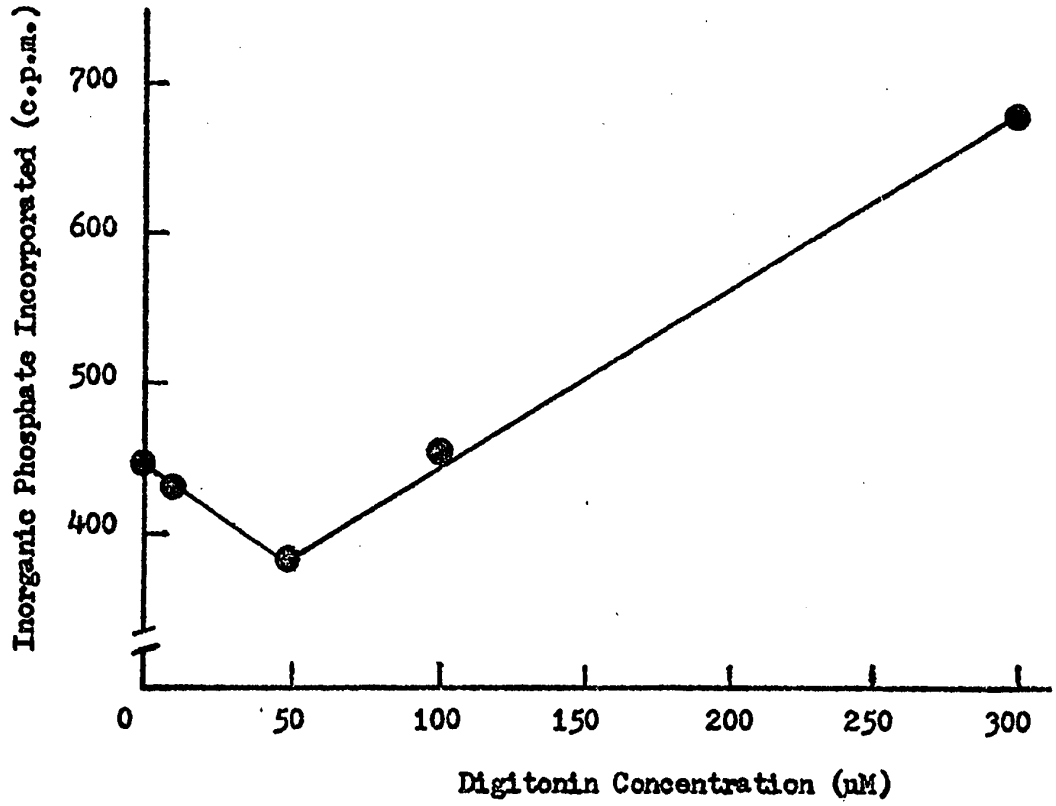


Figure 9: Effect of digitonin on rat liver mitochondrial inner membrane polynucleotide phosphorylase. Poly A phosphorolysis was measured in the standard condition, in the absence of Triton X-100, with the indicated digitonin concentrations.

this effect was not observed with the purified enzyme. At low concentrations of both fractions, below the lower limits indicated above, the assay was not proportional to enzyme concentration and this effect was not eliminated by Triton X-100 (Fig. 6). However, the detergent stimulated phosphorolysis by the inner membrane suspension 100% and by the partially-purified enzyme 30%.

There was a lag phase in the reaction catalyzed by either the crude or purified polynucleotide phosphorylase, but the measured rate of phosphorolysis with or without Triton X-100 was linear provided the time of incubation was between 45 and 120 minutes (Fig. 8).

Since digitonin is used in the preparation of the inner mitochondrial membranes, its effect on the phosphorolytic activity of the membrane-bound enzyme was studied. When assayed in the absence of Triton X-100, a slight inhibition was observed with digitonin concentrations below 100 μM and some stimulation at higher concentrations (Fig. 9). Dialysis of the washed inner membranes had no significant effect on their polynucleotide phosphorylase activity. The effect of digitonin on the enzyme in the presence of Triton X-100 was not studied.

All attempts to detect nucleoside diphosphate polymerization activity associated with the partially purified enzyme failed. Many combinations of the following parameters were tried using standard polymerization assay techniques (see p. 38) that would detect the formation of acid-insoluble, radioactive products from ^{14}C-labelled nucleoside diphosphates: (i) 1-5 mM-ADP, 5mM-CDP, 5mM-UDP; (ii) 1-8 mM-$MgCl_2$ and 0.2-8 mM-$MnCl_2$ (see p. 4);

(iii) pH 7.4, 7.8, 8.2, 8.6 and 9.0; (iv) the addition of: a) bovine serum albumin (100 and 200 µg/ml), b) cyclic AMP (5 µmole/ml), c) ApApA (100 and 200 µg/ml) (see p. 6), d) oligo- and polylysine and polyornithine (cf. Fitt and Wille, 1969a) (see p. 12), e) 10 mM-NaCl and f) cetyltrimethylammonium bromide. Since such methods fail to detect a very limited incorporation into acid soluble oligonucleotides, a search for such products was made. The experiment described in Fig. 10 shows that no detectable formation of oligonucleotides occurs with partially purified mitochondrial polynucleotide phosphorylase in the presence or absence of ApA. The enzyme preparation contained a nuclease that degraded ^{14}C-poly A to acid soluble products but chromatographic analysis of the latter (Fig. 11) showed that they were almost entirely oligonucleotides and that little or no AMP was formed. Thus, the failure to detect oligonucleotides in the experiment described in Fig. 10 was not due to their degradation to AMP by the contaminating nuclease, since the latter was clearly an endonuclease. It therefore appears that mitochondrial polynucleotide phosphorylase is devoid of polymerization activity, and in this respect, resembles the E. coli Q13 (Thang et al., 1969) and E. coli PR7 (Reiner, 1969a), polynucleotide phosphorylases that also lack polymerization and exchange activities.

The endonuclease activity present in the partially purified mitochondrial polynucleotide phosphorylase was more active in the optimal condition described for the ribonuclease isolated from rat liver mitochondria (Morais, Blackstein and Lamirande, 1967) than in the conditions used in Fig. 11. This ribonuclease was shown to

Figure 10: Chromatographic analysis of the reaction mixture from an attempted polymerization of ADP in the presence of mitochondrial polynucleotide phosphorylase. The reaction mixtures (0.1 ml) contained: 0.1 M-tris-HCl buffer, pH 7.8; 6 mM-$MgCl_2$; 1 mM-2-mercaptoethanol; 5 mM-^{14}C-ADP (656,000 c.p.m./ µmol); 0.1% (v/v) Triton X-100; 1.6 mM-ApA, where appropriate; partially purified particulate rat liver mitochondrial polynucleotide phosphorylase, 100 units of phosphorolysis activity. Reactions with and without ApA were performed, together with a similar control without enzyme but with ApA. Incubation was at 37° C for 1 h. The tubes were then allowed to stand in an ice-bath for 10 min. The particulate enzyme was removed by centrifugation in a clinical centrifuge (full speed for 2 min) and 40-50 µl samples of the supernatant were applied to a chromatogram (Whatman no. 1 paper). Authentic samples of the terminally-dephosphorylated oligonucleotides $(Ap)_nA$, n = 1-5, and of ATP, ADP and AMP were run in separate lanes on the paper, and the chromatogram was developed for 13 hours by descending chromatography in 1 M-ammonium acetate buffer, pH 7.1-95% (v/v) ethanol, 70:30 (v/v) (Thach, 1966). The chromatogram was dried with cool air and the standards were located under u.v. light. The lanes corresponding to the reaction mixtures and the control were then cut into strips 2.5 cm long and the radioactivity of the latter was determined. (●), control; (○), reaction mixture with ApA. (The reaction without ApA was similar, but is not shown.)

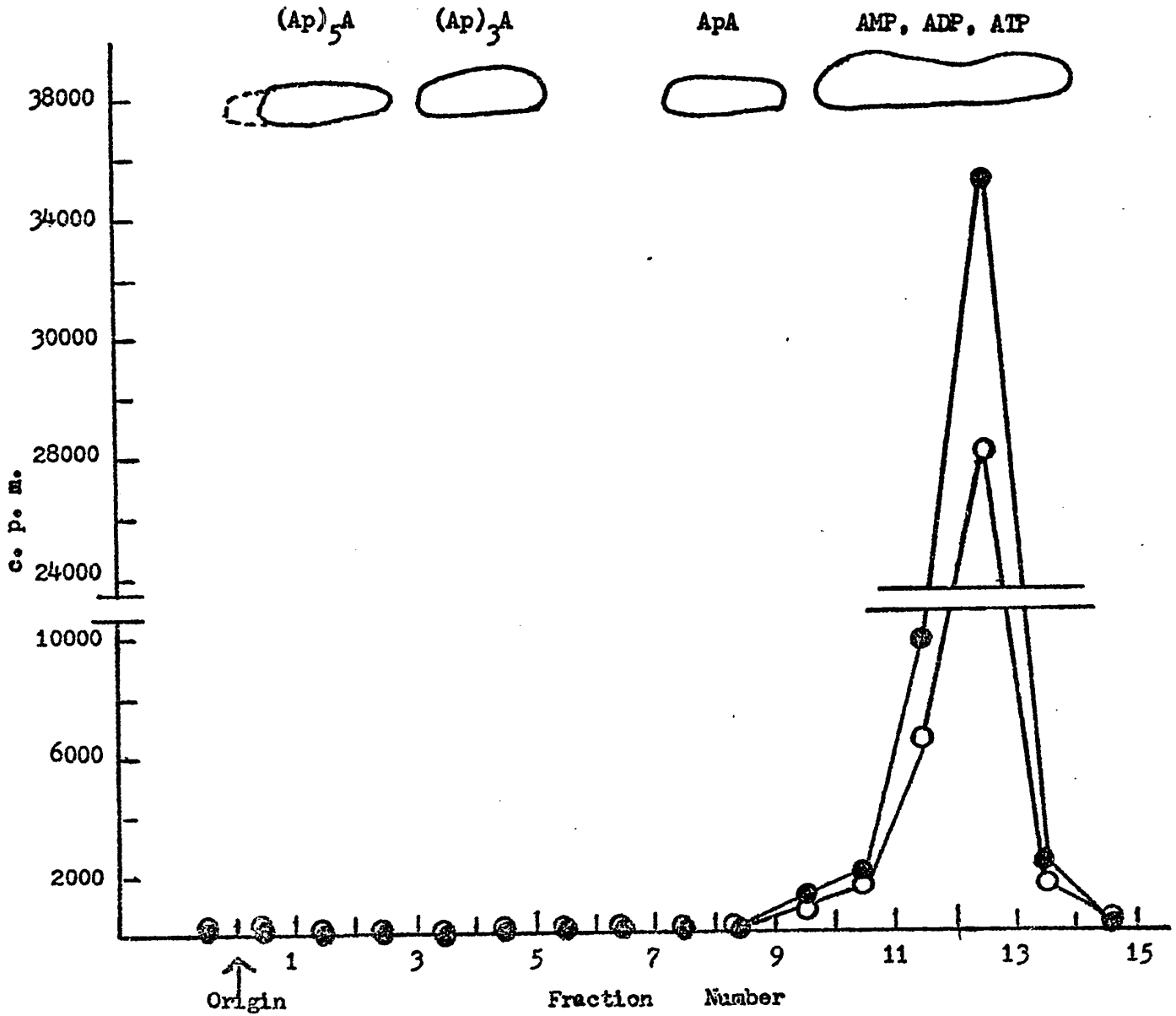

(Ap)5A
(Ap)3A
ApA
AMP, ADP, ATP
38000
34000
30000
28000
24000
10000
6000
2000
c. p. m.
Origin
1
3
5
7
9
11
13
15
Fraction Number

Figure 11: Chromatographic analysis of the products of degradation of ^{14}C-poly A by nucleases present as contaminants in the mitochondrial polynucleotide phosphorylase. The reaction mixture (0.1 ml) contained: 0.1 M-tris-HCl buffer, pH 7.8; 6 mM-$MgCl_2$; 1 mM-2-mercaptoethanol; ^{14}C-poly A, 1.5 mg/ml (168,000 c.p.m./mg); 0.1% (v/v) Triton X-100; partially purified (particulate) mitochondrial polynucleotide phosphorylase, 100 units of phosphorolysis activity. The control was a similar mixture lacking enzyme. Incubation, chromatgraphy (for 16 h) and subsequent operations were performed as described in Figure 10.(●), control;(○), reaction mixture.

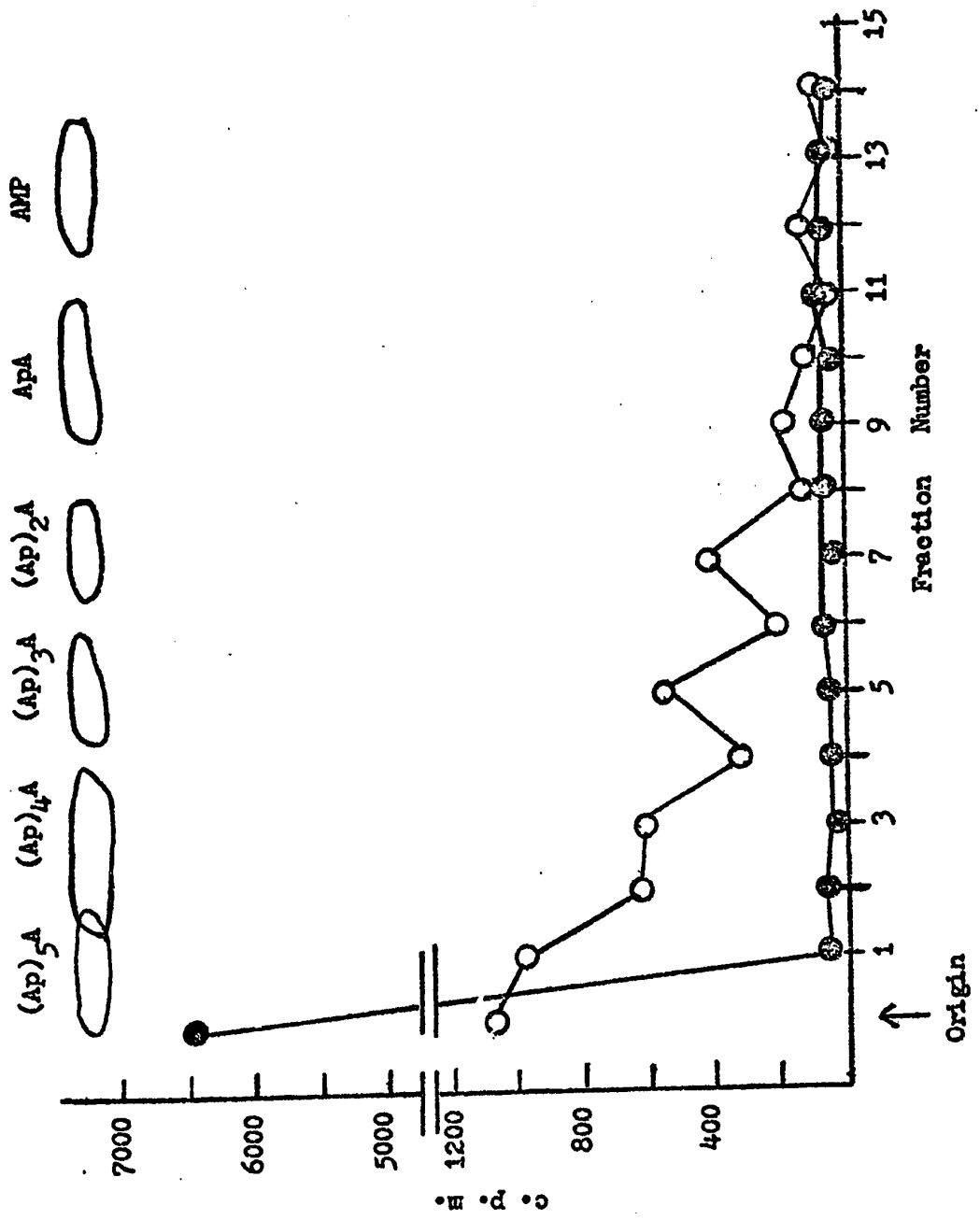

(Ap)5A
(Ap)4A
(Ap)3A
(Ap)2A
ApA
AMP
7000
6000
5000
1200
800
400
c. p. m.
Origin
1
3
5
7
9
11
13
15
Fraction Number

TABLE 9

Adenylate kinase activity of partially purified rat liver mitochondrial polynucleotide phosphorylase

The adenylate kinase was determined with 1mM ^{14}C-ADP and 29 µg enzyme protein as described in the Methods section.

Products	radioactivity (c.p.m.)	Percent of total radioactivity
ATP	13623	28
ADP	21169	43
AMP	14292	29

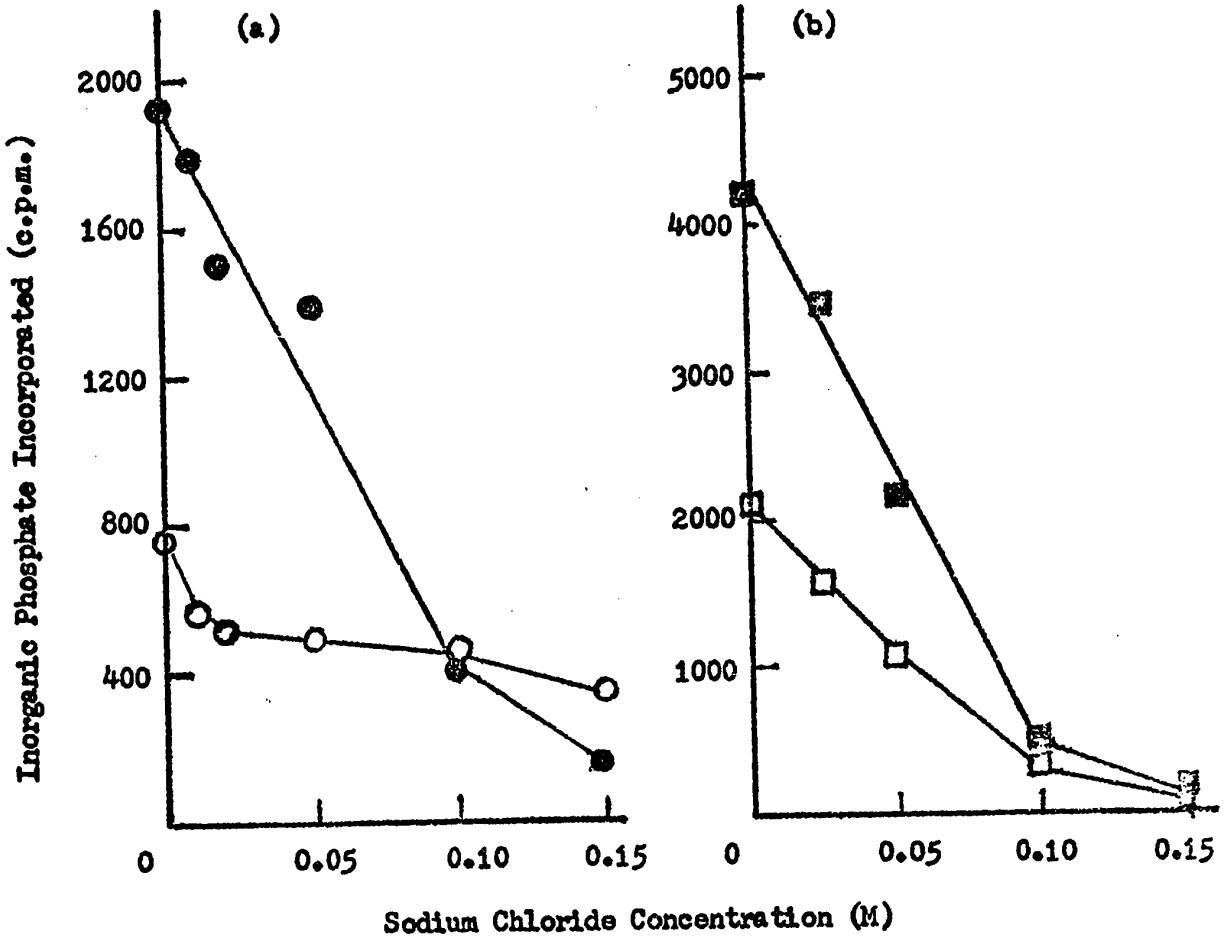


Figure 12: Effect of sodium chloride on the phosphorolysis and exchange activities of rat liver mitochondrial polynucleotide phosphorylase. (a) The activity of the partially purified enzyme was determined in the standard poly A phosphorolysis (●) and ADP/Pi exchange (○) assays at the indicated NaCl concentrations. (b) Poly A phosphorolysis activity of inner membrane was determined in the presence (■) and absence (□) of Triton X-100 at the indicated NaCl concentrations.

TABLE 10

Effect of ApA on phosphorolysis and exchange reactions of partially purified rat liver mitochondrial polynucleotide phosphorylase

Poly A phosphorolysis and the nucleoside diphosphate-inorganic phosphate exchange reactions were measured in the standard conditions, in the absence or presence of ApA (3.2mM).

Reactions	^{32}P-P_i incorporated (c.p.m.)		Percent inhibition by ApA
	- ApA	+ ApA	
poly A phosphorolysis	4092	2587	37
ADP/P_i exchange	1744	1080	38
dADP/P_i exchange	867	518	40

TABLE 11

Effect of detergent on the phosphorolysis and exchange reactions of partially purified rat liver mitochondrial polynucleotide phosphorylase

Poly A phosphorolysis and ADP/P_i exchange activites were assayed in the standard conditions described in the Methods section, with the indicated concentrations of detergents.

Detergents	Phosphorolysis ^{32}P-P_i incorporated (c.p.m.)	Residual activity (%)	ADP/P_i exchange ^{32}P-P_i incorporated (c.p.m.)	Residual activity (%)
(i) none	1317	100	564	100
(ii) Triton X-100				
0.1% (v/v)	1722	131	572	101
(iii) Sodium cholate				
0.1% (w/v)	932	71	566	100
0.2% (w/v)	513	39	505	90
(iv) Sodium dodecyl sulphate				
0.05% (w/v)	0	0	0	0
0.1% (w/v)	0	0	0	0

TABLE 12

Substrate specificity of exchange activity

Exchange activity was measured in the conditions of the standard ADP/P_i exchange using 0.5 µmole/0.1 ml of the indicated substrates and 30 µl/0.1 ml of the purified enzyme from preparation no. 2 (Table 1; 2.36 mg protein/ml). Controls without enzyme had a radioactivity of 63 ± 4 c.p.m.

Nucleotide	^{32}P-P_i incorporated (c.p.m.)	- control without substrate
none	120 (± 5)	-
ATP	1148	1028
ADP	3328	3208
AMP	93	0
dADP	2380	2260
UDP	129	9
CDP	240	120
GDP	116	0

degrade poly A to oligonucleotides with 5'-phosphate termini. Although the bulk of the activity was associated with the outer membrane and the space between the outer and inner-membrane of the mitochondria fractionated by the digitonin procedure, about 12% of the enzyme was still found in the inner membrane-matrix fraction (Morais, 1969) and could be responsible for the endonuclease activity that we observed.

The partially purified polynucleotide phosphorylase was also contaminated with adenylate kinase (Table 9). The amount of ATP is about the same as the amount of AMP produced (Table 9; cf. Table 1). This result confirmed the result obtained by phosphate determination that phosphatase capable of degrading ADP was absent in the partially purified enzyme.

The partially purified and membrane bound polynucleotide phosphorylase were inhibited to the same extent by NaCl (Fig. 12). This behaviour resembles that of the polynucleotide phosphorylases isolated from guinea-pig nuclei and rat liver nuclei (p. 133). Sucrose up to 0.1M had no effect on the activity of the membrane bound enzyme in the presence of Triton X-100.

The phosphorolysis reaction was inhibited about 60% by 3.2mM ApA (Table 10) and 0.2% (w/v) sodium cholate (Table 11). It was inhibited 100% by 0.05% (w/v) sodium dodecyl sulphate (Table 11).

NDP/P_i exchange activity

The partially purified polynucleotide phosphorylase was associated with a highly specific exchange activity (Table 12 and Fig. 13). It can be seen that, although ADP is the preferred subs-

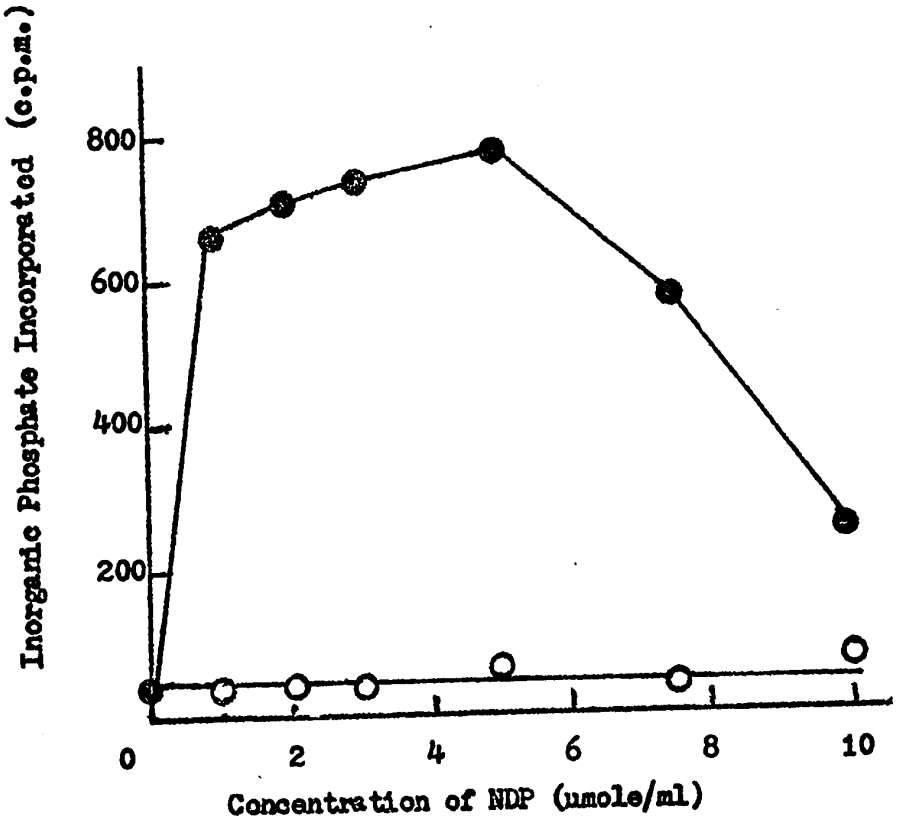


Figure 13: Substrate specificity of the NDP/Pi exchange activity associated with rat liver mitochondrial polynucleotide phosphorylase. The NDP/Pi exchange activity of the partially purified enzyme (Table 5, Preparation No. 1) was measured in the condition of the standard exchange assay at the indicated concentrations of the nucleoside diphosphates and with 17 units of ADP/Pi exchange activity/assay. The control contained the appropriate concentration of each nucleotide, but no enzyme and their values were subtracted from the assay values. ADP,(●); CDP, GDP and UDP,(○) (each point is the average of the almost identical values with these three substrates).

trate, ATP and dADP were also effective. UDP and GDP were not substrates. The data presented in Fig. 12 shows that the specificity for ADP was not due to a difference in the substrate optimum in the case of the other ribonucleoside diphosphates. A limited, but reproducible, CDP/P_i exchange was observed (Table 12) in one enzyme preparation (Table 5, preparation 2) using three times as many ADP/P_i exchange units in the assay as in the experiment described in Fig. 13 (using preparation 1, Table 5). The former enzyme preparation contained twice as many ADP/P_i exchange units as the latter (Table 5). The ATP/P_i exchange activity observed (Table 12) was not due to ADP contamination in the ATP solution. The ATP used was shown to contain less than 0.9% (mole/mole) of ADP by t.l.c. (Randerath, 1964) followed by elution and spectrophotometric estimation of the separated nucleotides. The enzyme preparation also catalysed dADP/P_i exchange.

The ADP/P_i exchange activity of the partially purified enzyme had a pH optimum at 7.8 (Fig. 3), different from that of the phosphorolysis reaction (Fig. 3). The pH optimum of the exchange reaction in the crude inner membrane preparation was not determined.

The enzyme required divalent cation for activity, with an optimum concentration of 6mM for Mg^{2+} and a plateau between 0.8 and 4mM for Mn^{2+} (Fig. 4). At low divalent cation concentration, Mn^{2+} is the preferred cation. Ca^{2+} could not replace Mn^{2+} or Mg^{2+}.

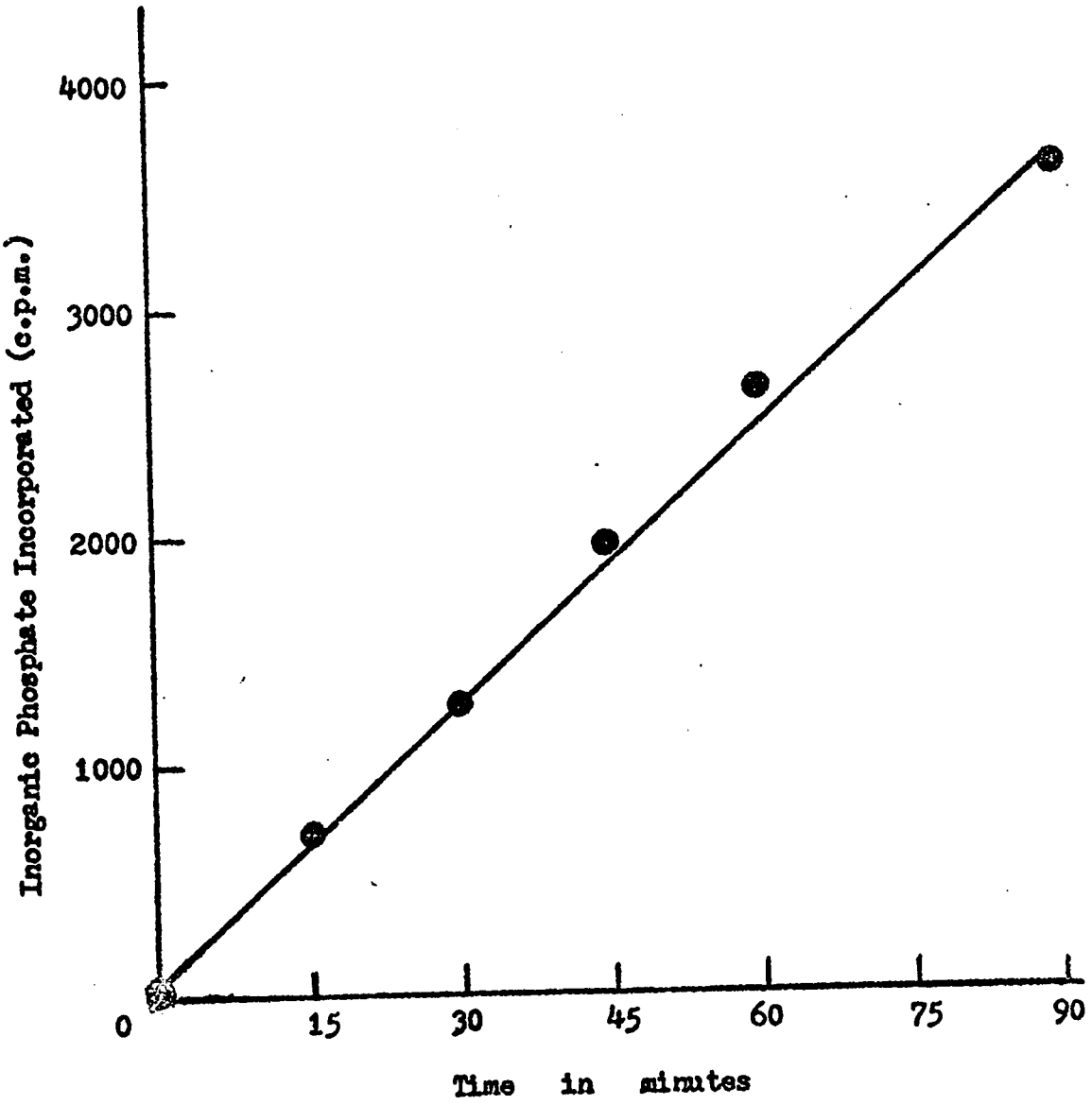


Figure 14: Time course of ADP/Pi exchange reaction associated with partially purified rat liver mitochondrial polynucleotide phosphorylase. The ADP/Pi exchange activity was measured in the standard assay conditions.

In the conditions of the standard ADP/P_i exchange assay, the enzyme saturation curve was linear at low enzyme concentration, unlike the phosphorolysis assay described above, and incorporation of P_i into nucleoside diphosphate was linear for 90 min up to 50 units of ADP/P_i exchange units (Fig. 14).

The ATP/P_i exchange activity was not studied further, but some studies had been made on the dADP/P_i exchange activity. However, the stock solution of dADP used for these studies was contaminated by ADP (2% mole/mole). The E. coli (Kaufman and Littauer, 1969; Bon et al., 1970) and M. lysodeikticus (Chou and Singer, 1971a) polynucleotide phosphorylase catalyse a slow dADP/P_i exchange reaction in the presence of oligonucleotide (see p. 19 for discussion). This requirement for oligonucleotide may be replaced to some extent by ADP. The dADP/P_i exchange reaction observed by Kaufman and Littauer (1969) in the absence of oligonucleotides was thought to be due to ADP contamination (Chou and Singer, 1971a). The dADP/P_i exchange reaction observed in the partially purified polynucleotide phosphorylase may not be due to ADP stimulation. Preliminary results show that addition of ADP in the reaction mixture did not stimulate dADP/P_i exchange and oligonucleotide (ApA) inhibited the reaction (Table 11). Final confirmation of the presence of a genuine dADP/P_i exchange reaction in the mitochondria will have to await further studies with purified substrate free from ADP.

Since the exchange and phosphorolysis reactions were inhibited about 40% by the addition of ApA (2 mg/ml; 3.2mM) to the appropriate assay medium (Table 10), and the preparation did not catalyse ADP polymerization, it appears that the exchange reaction cannot depend

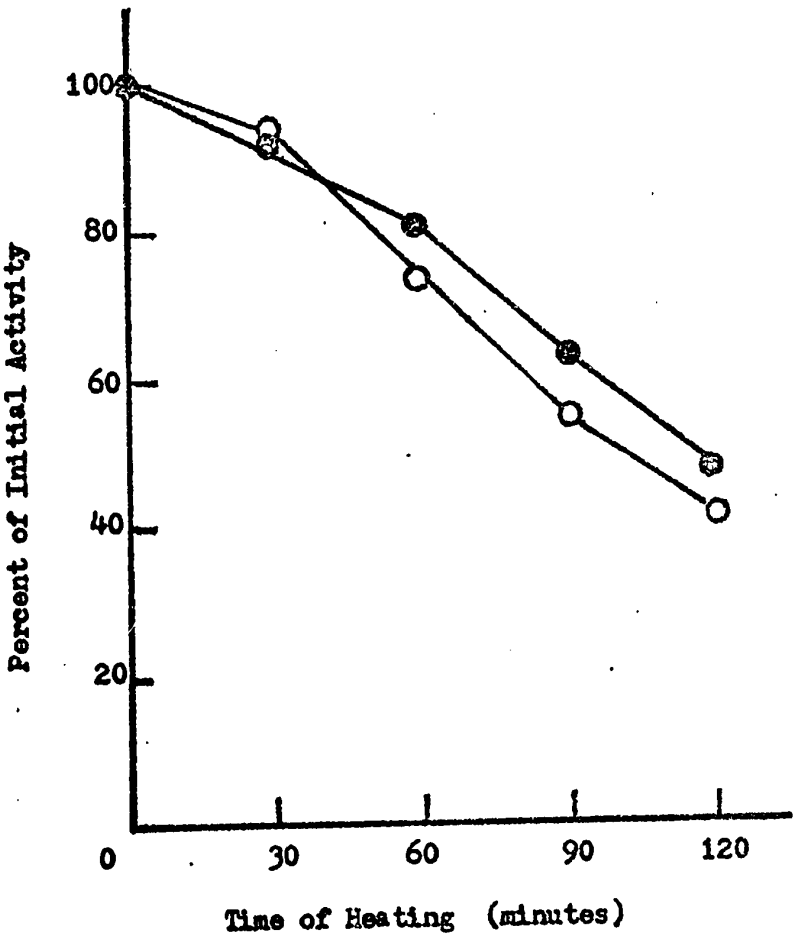


Figure 15: The stability (37°) of the phosphorolysis and ADP/Pi exchange activity of partially purified rat liver mitochondrial polynucleotide phosphorylase. A suspension of partially purified polynucleotide phosphorylase was heated at 37° and, at the times indicated, samples were withdrawn and assayed for poly A phosphorolysis (●) and ADP/Pi exchange activity (○).

on a reversible phosphorolysis-polymerization reaction taking place in near equilibrium conditions. In the case of the well-studied bacterial polynucleotide phosphorylases that catalyse both phosphorolysis and polymerization, such a mechanism is believed to be responsible for NAD/P_i exchange; and oligonucleotides with free 3'-OH terminal stimulate the reaction, or may be required absolutely in the case of dADP (see p. 19 for discussion).

The phosphorolysis and exchange activities were inhibited by sodium chloride (Fig. 12a). Although the exchange reaction was somewhat less sensitive to changes in the ionic strength of the medium, they had very similar stabilities at 37° (Fig. 15). These observations, together with the similarity of the effect of ApA on the two reactions (Table 10) suggest that both activities are to be due to one enzyme. Nevertheless, the restricted specificity of the exchange reaction, with its marked preference for adenine nucleotides, is in notable contrast with that of the phosphorolytic reaction which occurs readily with poly A, poly C, and poly U (Table 7), and supports the opposite view, i.e. that two different enzymes are responsible for exchange and phosphorolysis respectively. This latter possibility appears to be confirmed by the behaviour of the two activities during purification (see above) and the fact that the most highly-purified polynucleotide phosphorylase preparations (Table 5, no. 3) were devoïd of detectable ADP/P_i-exchange activity.

Further, the two activities of the purified enzyme, were affected differently by Triton X-100 and sodium cholate: 0.1%

(v/v) Triton X-100 stimulated poly A phosphorolysis 30% while it had no effect on the rate of ADP/P_i exchange; in contrast, phosphorolysis was inhibited 30% by 0.1% (w/v) and 62% by 0.2% (w/v) sodium cholate, while the lower concentration did not affect the ADP/P_i exchange activity and the higher concentration of detergent caused only a 10% inhibition (table 11). Both activities were inhibited totally by 0.05% (w/v) sodium dodecyl sulphate.

All the phosphatases active with nucleoside diphosphates are removed during the RNA-gel/DEAE-cellulose column step of the purification and therefore cannot be responsible for the exchange reaction, so we conclude that the mitochondrial inner membrane contains both an enzyme capable of catalysing ADP/P_i-exchange and a polynucleotide phosphorylase with phosphorolytic activity only.

2. Animal nuclear polynucleotide phosphorylase

(i) Isolation and purification of guinea-pig liver and rat liver nuclear polynucleotide phosphorylase

As a first approach to demonstrate the presence of polynucleotide phosphorylase in animal tissues, the data reported by Hilmoe and Heppel (1959) using extracts from guinea-pig nuclei were re-examined. Their results have been discussed in detail in the Introduction (p. 28). Very little detail was given in this paper and the procedures used for the isolation of nuclei and nuclear extract by the authors are not known. Guinea-pig liver nuclei were therefore isolated by the 2.2M-sucrose procedure described in the Methods section (Method xvi). The nuclei were extracted with 0.25M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 as described in the Methods section (method xviii). When the crude extract was assayed in the conditions described by Hilmoe and Heppel (1959), charcoal adsorbable radioactive material could be obtained. However, the non-enzymic blank was high and variable. This was due to the use of 0.1M-phosphate buffer, in the assay, which required the addition of large amounts of ^{32}P-labelled inorganic phosphate (ten times more radioactivity was require for this assay as the standard phosphorolysis assays I normally used). For this reason, the phosphate buffer was replaced initially by imidazole buffer of the same pH (7.2). However, more detailed studies showed that the purified enzyme was most active between pH 8.6 and 9.2 and tris-HCl buffer, pH 8.7 was then used in the routine assay for the guinea-pig liver nuclear polynucleotide phosphorylase.

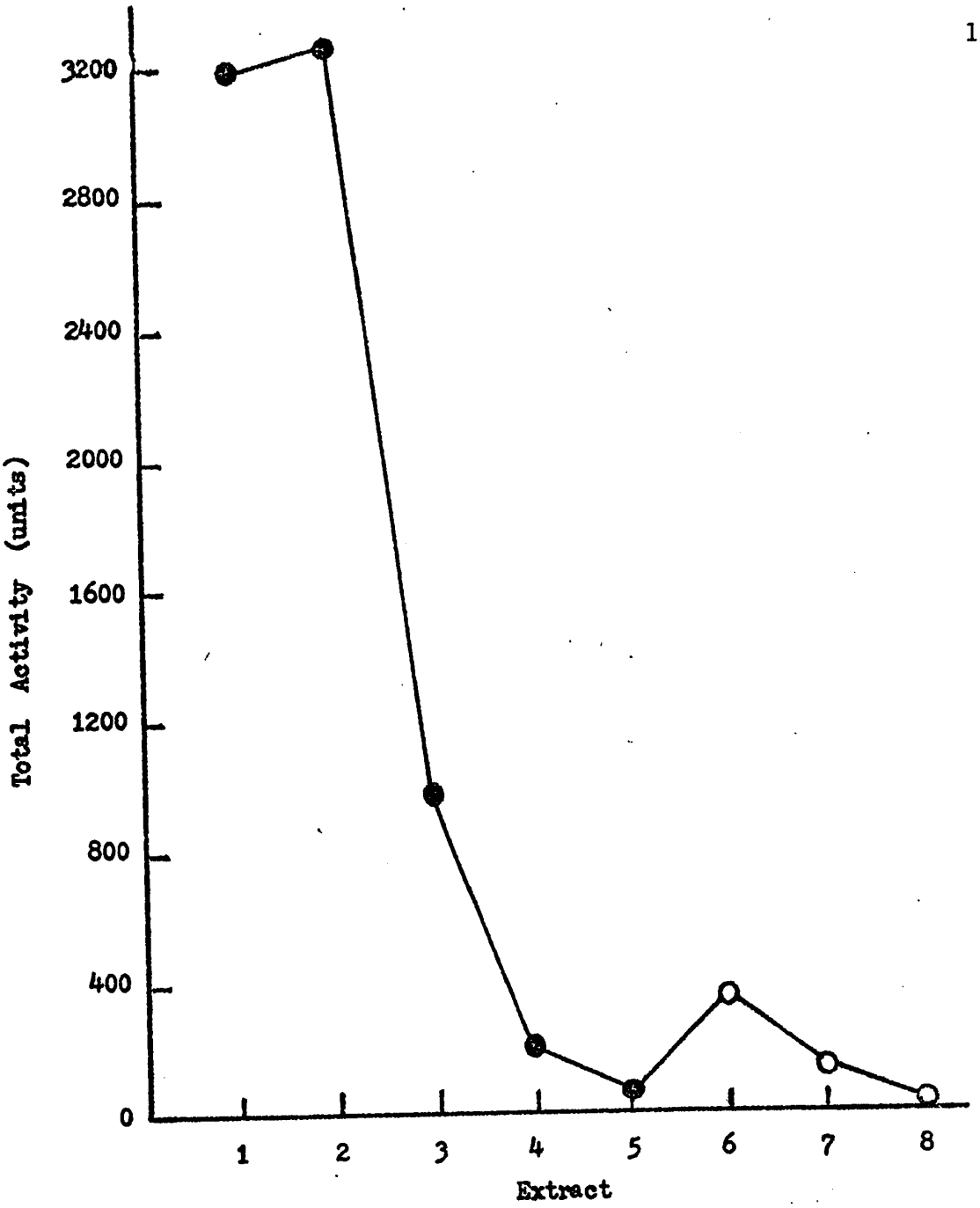


Figure 16: Extraction of guinea-pig liver nuclei with isotonic sucrose and NaCl solutions. The crude nuclei, prepared by the 2.2 M-sucrose procedure (method xvi), were extracted five times with 0.25 M-sucrose-1 mM-2-mercaptoethanol-10 mM tris-HCl buffer, pH 7.4, and then three times with 0.14 M-NaCl- 1 mM 2-mercaptoethanol-2 mM-EDTA, pH 7.4. The individual extracts were dialysed overnight against 1 mM-2-mercapto-ethanol-2 mM-EDTA, pH 7 (20 vol.) and assayed for phosphorolytic activity. Sucrose extracts,(●); saline extracts,(○).

Imidazole buffer, pH 7.2 was also used in the DEAE-cellulose column chromatographic step in the purification of the guinea-pig liver nuclear polynucleotide phosphorylase (Method xix). However, imidazole is unstable and turns yellow after several months of storage. For this reason, in the purification of the rat liver polynucleotide phosphorylase, the imidazole buffer was replaced by tris-HCl buffer, pH 7.4 (method xviii).

The bulk of the work on animal nuclear polynucleotide phosphorylase was done with the guinea-pig liver enzyme. There was no problem in detecting polynucleotide phosphorylase activity in a crude extract of fed guinea-pig liver nuclei by the standard poly A phosphorolysis assay described for the guinea-pig liver nuclear enzyme. There was no detectable activity when fasted animals were used or when the animal had just arrived from the suppliers (from Montreal). This problem has not yet been examined further, and fed, properly rested animals were used throughout the work on animal polynucleotide phosphorylase.

Purification of guinea-pig and rat liver nuclear polynucleotide phosphorylase

Guinea-pig and rat liver nuclei were prepared by the 2.2M-sucrose procedure (method xvi) and the polynucleotide phosphorylase was extracted as described in methods xviii and xix. Fig. 16 shows the extraction of guinea-pig liver nuclear polynucleotide phosphorylase by sucrose and saline. It can be seen that two fractions could be clearly separated by the extraction procedures. Further extraction of the nuclear pellet with 1M-sodium chloride solution failed to yield additional polynucleotide phosphorylase.

TABLE 13

Purification of guinea-pig liver and rat liver nuclear polynucleotide phosphorylase

The enzyme was prepared as described in the Methods section. One unit is the amount of enzyme that catalyses the incorporation of 1nMole of ^{32}P-Pi into ADP/h in the conditions of the poly A-phosphorolysis assay.

Exp.	Step	Sucrose fraction			Saline fraction		
		Units	Protein (mg)	Sp. activity (units/mg)	Units	Protein (mg)	Sp. activity (units/mg)
(a) Guinea-pig liver nuclei							
	1. Crude extract	7800	194	40	482	6.5	74
	2. pH 5.7	11200	66	170	228	0.9	253
	3. DEAE-cellulose 0.3M-NaCl eluate	3800	5.6	680	-	- -	-
(b) rat liver nuclei							
	1. crude extract	1330	103	13	-	-	-
	2. pH 5.6	2052	42	49	-	-	-
	3. DEAE-cellulose 0.3M-NaCl eluate	1242	2.6	478	-	-	-

Polynucleotide phosphorylase was also extracted by sucrose from rat liver nuclei but, in this case, no further activity was recovered by subsequent saline extraction.

Polynucleotide phosphorylase activity could be easily detected in crude sucrose extracts of liver nuclei from fed guinea-pig when the assay mixture was incubated at 37° for 1 h. There was little detectable activity when similar crude rat liver nuclear extract was assayed for this length of time, but definite polynucleotide phosphorylase activity could be measured when the assay was performed for three hours or longer. Preliminary work with the rat liver nuclear enzyme showed that when the dialysed crude extract was heated at 37° for 2.5 h, definite and reproducible polynucleotide phosphorylase activity could be detected in a 1 h incubation assay. This may be due to the inactivation of phosphatases that degrade ADP produced in the phosphorolysis reaction. This heating step was not used in the purification procedure because most of the interfering enzymes seemed to be removed in the subsequent pH precipitation step, as shown by the increase in total polynucleotide phosphorylase activity (Table 13(b)). A similar increase in total polynucleotide phosphorylase activity was also seen during the purification of the guinea-pig liver nuclear enzyme (Table 13(a)).

The sucrose and saline extracts of the guinea-pig liver nuclear enzymes were precipitated when the pH was lowered to pH 5.7 (with 1M-sodium acetate buffer, pH 5.3). The sucrose extract of the rat liver nuclear enzyme was not precipitated from the sucrose extracts until the pH was lowered to 5.6. The enzymes

TABLE 14

Lipids associated with the particulate guinea-pig liver nuclear polynucleotide phosphorylase

The lipids were extracted and separated as described in the Methods.

Spot No.	Standards		Extract	
	Lipid	R_F	R_F	Intensity
1	Lysophosphatidylcholine	0.096	0.097	Faint
2	Phosphatidylcholine	0.31	0.30	Dark
3	Phosphatidylethanolamine	0.62	0.61	Medium
4	-	-	0.72	Very faint
5	Cholesterol	0.93	0.94	Dark
6	Mixture of mono-, di- and triglycerides	0.98	0.98	Very dark

were further purified by DEAE-cellulose column chromatography as described in the Methods. The 0.3M-NaCl eluates from the column were clear or slightly cloudy,but white particles, containing all the polynucleotide phosphorylase activity, formed during removal of salt by dialysis. The final particulate partially purified sucrose extract will be referred to as the "sucrose fraction". The saline extracts, after pH 5.7 precipitation, was redissolved in 1mM-2-mercaptoethanol-2-mM-EDTA (1 ml) and dialysed against the same buffer overnight. This fraction was also obtained in particulate form and will be referred to as the "saline fraction".

The bulk of the data presented below were obtained with the sucrose fraction of the guinea-pig liver nuclear enzyme. Some of the properties of the saline fraction and the rat liver nuclear sucrose fraction were also studied in order to compare them with those of the guinea-pig liver nuclear sucrose fraction.

The particulate sucrose fraction (of the guinea-pig liver nuclear enzyme) dissolved very quickly on the addition of sodium dodecyl sulphate (5mM). This suggested that the enzyme might be bound to membrane particles. Lipid analysis showed that the particles contained lipids (phospholipids and neutral lipids) characteristic of membranes (Table 14). When the particulate enzyme was solubilized with 5mM sodium dodecyl sulphate and the detergent removed by dialysis, a clear solution was obtained, but the enzyme was completely inactivated. The addition of magnesium chloride to this clear solution (final concentration, 10mM) produced an inactive white precipitate similar in appearance

to the original particles. Engelman (1968) has shown that the membrane of Mycoplasma laidlawii can be solubilized by 10mM-sodium dodecyl sulphate and that it is reconstituted during dialysis against 20mM-magnesium chloride.

In order to verify if the enzyme was in a particulate form in the crude extract, a dialysed sucrose extract was centrifuged, first at 40,000 g for 20 min to remove visible solids, and then at 78,000 g (30,000 r.p.m., Beckman type 30 rotor). After 1.25 h 53% of the activity had sedimented and this was raised to 69% after 3.25 h, suggesting that the enzyme was attached to membrane fragments of various sizes. This would explain the loss of about 65% of the activity during DEAE-cellulose chromatography (Table 13(a)), when the largest particles would collect on the top surface of the ion-exchanger. Further, since all the activity eluted from the column was recovered in particulate form after removal of sodium chloride by dialysis, and the saline fraction was also solid, it seems certain that all the extracted enzyme was membrane bound, and must have broken away from the nuclear preparations during extractions.

The nuclear pellet, as prepared by the 2.2M-sucrose procedure, was very small and swelled considerably during the extractions with 0.25M-sucrose. After extraction, all the nuclei were seen to be broken when examined by phase-contrast microscopy. The nuclei in 2.2M-sucrose appeared very small, as seen under a phase-contrast microscope, but swelled considerably when the nuclei were transferred to 0.25M-sucrose (without homogenization). Most of these nuclei were still intact. From these observations, it

TABLE 15

Lipids associated with the particulate rat liver nuclear polynucleotide phosphorylase

The lipids were extracted and separated as described in the Methods.

Spot No.	Standards		Extract	
	Lipid	R_F	R_F	Intensity
1	Lysophosphatidylcholine	0.10	0.12	Faint
2	Phosphatidylcholine	0.35	0.36	Faint
3	-	-	0.47	Faint
4	Phosphatidylethanolamine	0.62	0.62	Medium
5	Cardiolipin	0.82	0.85	Medium
6	-	-	0.98	Dark

seems certain that the nuclei were broken during the extraction, involving sudden change in osmotic pressure combined with homogenization.

The purified particulate enzyme (sucrose fraction) contained RNA (0.1 mg/mg protein) but no detectable DNA.

The purified particulate rat liver nuclear polynucleotide phosphorylase also contained lipids characteristic of membranes (Table 15). The lipid composition of the rat liver nuclear enzyme is similar to that of the guinea-pig liver nuclear enzyme (Table 14), with the exception of a phospholipid spot with similar R_F value as standard beef heart cardiolipin (Table 15). The presence of this phospholipid was confirmed with two separate enzyme preparations. Cardiolipin is thought to be found exclusively in the mitochondria of rat liver (Getz, 1970), so its presence in the rat liver nuclear enzyme preparation suggested that the enzyme might be mitochondrial in origin and further work on this topic will be presented later.

Enzymic properties of guinea-pig liver and rat liver nuclear polynucleotide phosphorylase

Initially, the guinea-pig liver nuclear polynucleotide phosphorylase was eluted stepwise by 0.1M and 0.2M sodium chloride instead of 0.3M sodium chloride in the DEAE-cellulose chromatographic step. These stepwise elutions did not yield sufficient enzyme for detailed study of its properties and it is not certain whether the fractions were due to different isozymes or to the same enzyme attached to different sized membrane particles. The

TABLE 16

Specificity of guinea-pig liver polynucleotide phosphorylase

In method A the reaction mixture (0.3 ml) contained: 0.1M-imidazole-HCl buffer, pH 7.2; 10mM-$MgCl_2$; 1mM-2-mercaptoethanol; 10mM-$K_2H^{32}PO_4$ (about 30000 c.p.m./μmol); poly A or poly C, 1.5 mg/ml; fraction E_1, 92.5 μg/ml, or fraction E_2, 65 μg/ml (in one case fraction E1, 46 μg/ml, and fraction E_2, 32.5 μg/ml, together), or saline extract (0.74 mg/ml). Toluene was added to prevent microbial contamination. Incubation was at 37° for 12 h. Charcoal (1%, w/v) in 2.3% (w/v) $HClO_4$ (1 ml) was added. The mixture was kept at 0° for 10 min, and 1.25mM-KH_2PO_4 in 1% (w/v) $HClO_4$ (2 ml) then added. The charcoal was collected and washed five times with 1.25mM-KH_2PO_4 in 1%(w/v) $HClO_4$ (3 ml) and three times with water (3 ml). It was transferred with water (1 ml) to a Pasteur pipette fitted with a cotton-wool plug covered with cellulose powder. The nucleotides were eluted with 2% (w/v) NH_3 in 50% (v/v) ethanol. The eluate was evaporated to about 50 μl under partially reduced pressure at room temperature and applied, together with non-radioactive NMP, NDP and NTP (0.01 μmol of each), to a t.l.c. plate coated with Serva TLC-DEAE cellulose. The chromatogram was developed with 4% (w/v) formic acid (Fahn, Albers & Koval, 1965). The nucleotides were located with u.v. light and the spots were scraped off and the radioactivities counted (liquid scintillation). A control without enzyme was run exactly as above and the results were corrected accordingly. In method B the reaction and control mixtures (0.1 ml) had the same composition as in method A except that they contained (i) non-radioactive K_2HPO_4 and (ii) 1mM-14C-AMP, 14C-ADP or 14C-ATP instead of polyribonucleotide. After 12 h at 37° samples (50 μl) were applied directly to t.l.c. plates, and the spots were separated and the radioactivities counted as above. The table shows the radioactivities (c.p.m.) found in NMP, NDP and NTP after incubation in the indicated conditions. The values in parentheses represent the radioactivities present in the compounds as percentages of the total radioactivity applied to the chromatogram.

Expt.	Enzyme	Substrate	P_i	Radioactivity products [c.p.m. (%)] NTP	NDP	NMP
Method A						
a	E_1	Poly A	^{32}P-P_i	317 (2.2)	13168 (93)	565 (4)
b	E_2	Poly A	^{32}P-P_i	186 (1.2)	13240 (88)	1509 (10)
c	$E_1 + E_2$	Poly C	^{32}P-P_i	114 (1.1)	8923 (88)	1112 (11)
d	Saline extract	Poly A	^{32}P-P_i	83 (4.8)	1605 (92)	55 (3.2)
Method B						
e	E_1	^{14}C-ATP	P_i	15686 (83)	2192 (12)	987 (5)
f	E_2	^{14}C-ATP	P_i	12929 (86)	1153 (8)	917 (6)
g	E_1	^{14}C-ADP	P_i	3828 (13)	14624 (50)	11067 (37)
h	E_2	^{14}C-ADP	P_i	3203 (13)	14064 (61)	6503 (26)
i	E_1	^{14}C-AMP	P_i	0 (0)	682 (4)	16588 (96)
j	E_2	^{14}C-AMP	P_i	1 (0)	70 (0.5)	12422 (99.5)

product specificity of the phosphorolysis reaction was initially studied with these enzymes (Table 16). The conditions used for the product specificity study shown in Table 16 resemble closely those used by Hilmoe and Heppel (1959) except that imidazole buffer replaced phosphate buffer. Enzymes E_1 and E_2 were particulate enzymes obtained after dialysis of the 0.1M and 0.2M sodium chloride eluates, respectively, of the DEAE-cellulose column. Table 16 (Experiments a, b and c) shows that nucleoside diphosphate is the major radioactive product formed during incubation of either E_1 or E_2 with polyribonucleotides and ^{32}P-labelled inorganic phosphate. Little nucleoside triphosphate is formed. The amount of radioactive nucleoside monophosphate produced is low, and probably less than shown: some hydrolysis of $ND^{32}P$ to NMP and ^{32}P-P_i occurred during evaporation of the final ammoniacal ethanol solution, and the thin layer chromatographic system does not separate P_i from AMP or CMP.

Four reactions might lead to formation of NDP from polyribonucleotides or their conceivable breakdown products in these conditions: (i) hydrolysis of NTP; (ii) phosphorylaton of NMP; (iii) the action of an adenylate kinase-like enzyme on a mixture of NMP and NTP; (iv) phosphorolysis catalysed by polynucleotide phosphorylase. Reactions (i) and (ii) are excluded by the results in Table 16 which show that the amount of ^{14}C-ADP formed from ^{14}C-ATP (Expts. e and f) or ^{14}C-AMP (Expts. i and j) was insufficient to account for the phosphorolysis results (Expts. a, b and c). The amount of ^{14}C-ATP formed from ^{14}C-ADP (Table 16, Expts. g and h) indicates that although some adenylate kinase

TABLE 17

Product specificity of guinea-pig liver and rat liver nuclear polynucleotide phosphorylase

(a) Guinea-pig liver nuclear polynucleotide phosphorylase. The reaction mixture (0.2 ml) contained: 0.1M-tris-HCl buffer, pH 8.7; 4mM-$MgCl_2$; 1mM-2-mercaptoethanol; 10mM $K_2H^{32}PO_4$ (about 100,000 c.p.m./μmole); poly A, 1.5 mg/ml; enzyme. Incubation was at 37° for 2.5 h. Charcoal (1%, w/v) in 2.3% (w/v) $HClO_4$ (1 ml) was added. The charcoal was washed, the nucleotides eluted and separated as described in Table 16. A control without enzyme was carried out and the radioactivity corresponding to each nucleotide area on the TLC chromatogram was subtracted.

		Radioactivity products [c.p.m. (%)]		
Enzyme	Substrate	ATP	ADP	AMP
purified sucrose fraction	poly A + ^{32}P - P_i	36 (1.9)	1704 (89)	178 (9.3)

(b) Rat liver nuclear polynucleotide phosphorylase. The reaction conditions and procedures were as described for (a) above, except: 0.1M-tris-HCl, pH 7.8 and 6mM-$MgCl_2$ were used.

		Radioactivity products [c.p.m. (%)]		
Enzyme	Substrate	ATP	ADP	AMP
purified sucrose fraction	poly A + ^{32}P - P_i	82 (1.8)	3606 (80)	827 (18)

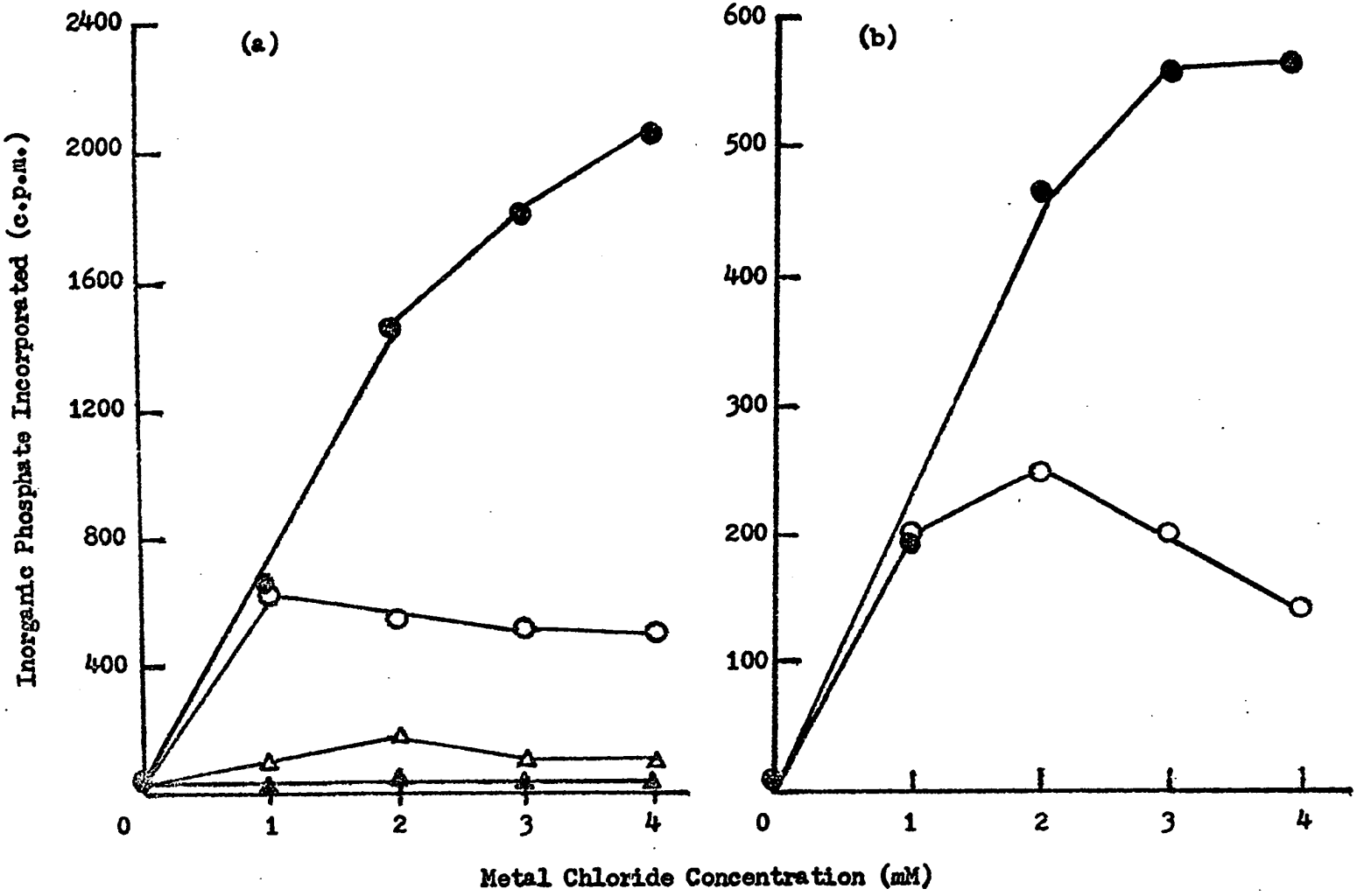


Figure 17: Influence of divalent cations on the phosphorolytic activity of guinea-pig liver polynucleotide phosphorylase. The (a) sucrose and (b) saline fractions were assayed for poly A phosphorolysis activity using the indicated concentrations of $MgCl_2$ (●), $MnCl_2$ (○), $CoCl_2$ (△), $ZnCl_2$ and $CaCl_2$ (▲).

was present it was insufficient to account for the phosphorolysis results even if both substrates could be formed. Reaction (iii) is therefore excluded. It can also be seen (Table 16, Expts. g and h) that a phosphatase able to hydrolyse ^{14}C-ADP to ^{14}C-AMP was still present. It would be expected to destroy part of the ^{32}P-NDP formed during phosphorolysis of polyribonucleotides, so that the measured amount of NDP produced (Table 16, Expts. a, b and c) was certainly lower than the true value. Thus the results in Table 16 prove that fractions E_1 and E_2 contained a polynucleotide phosphorylase.

The experiments presented above were not done at the optimal conditions for the guinea-pig liver nuclear polynucleotide phosphorylase. Table 17(a) shows that phosphorolysis of poly A by the purified particulate enzyme, under optimal conditions, also produced ADP as the major product and that very little ATP was formed. Similar results were obtained with the purified rat liver enzyme (Table 17(b)). These results prove that the isolated particulate enzymes from guinea-pig and rat liver nuclei contained polynucleotide phosphorylase.

Fig. 17 shows the effect of divalent cations on the phosphorolytic activity of the sucrose and saline fractions of guinea-pig liver nuclear enzymes. Both enzymes required Mg^{2+}, although Mn^{2+} could replace the latter to some extent. The sucrose fraction was slightly active in the presence of Co^{2+}, but Ca^{2+} and Zn^{2+} were ineffective (the last three cations were not tried with the saline fraction). These results are in general agreement with the known metal requirements of bacterial polynucleotide phospho-

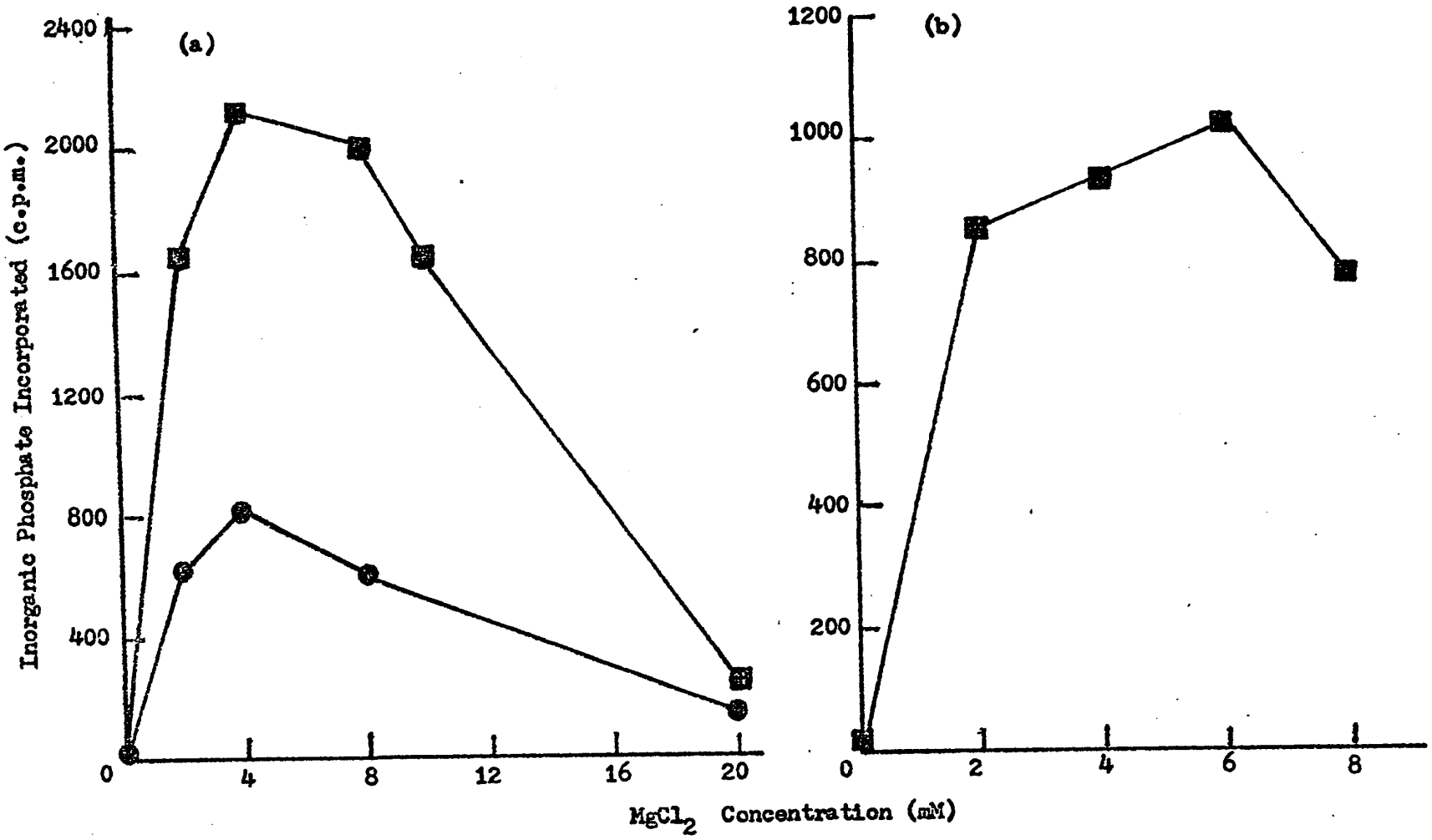


Figure 18: Influence of Mg^{2+} concentration on poly A phosphorolysis by guinea-pig and rat liver nuclear polynucleotide phosphorylase. Enzymatic activities of the sucrose and saline fractions were determined in the standard phosphorolysis conditions at the indicated Mg^{2+} concentrations. (a) guinea-pig liver; (b) rat liver; Sucrose fraction,(■); saline fraction,(●).

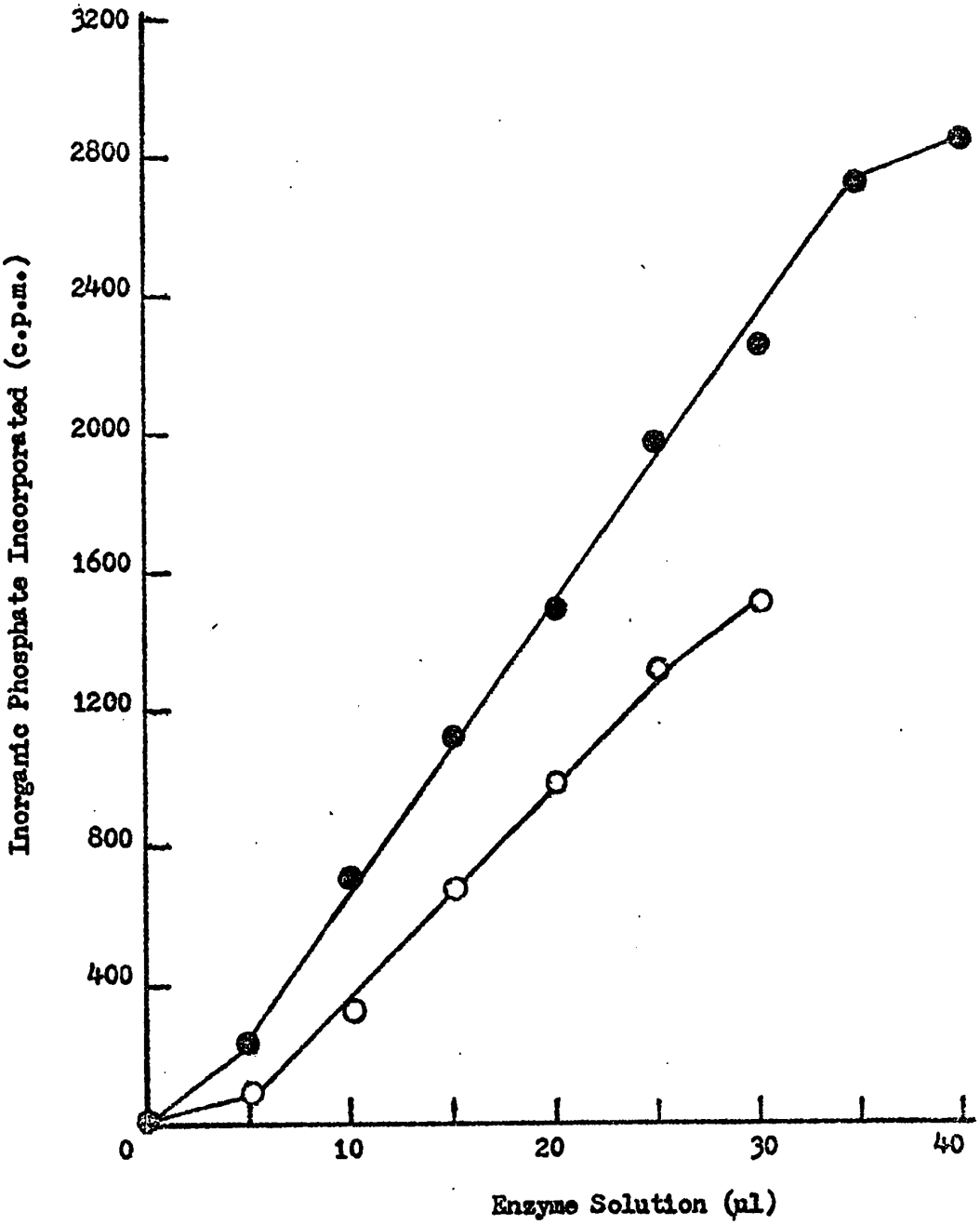


Figure 19: Enzyme-saturation curve for guinea-pig and rat liver nuclear polynucleotide phosphorylase. Phosphorolysis activity was measured in the standard assay with the indicated volumes of enzyme suspension. Guinea-pig (928 units/ml), (●); rat (790 units/ml), (○).

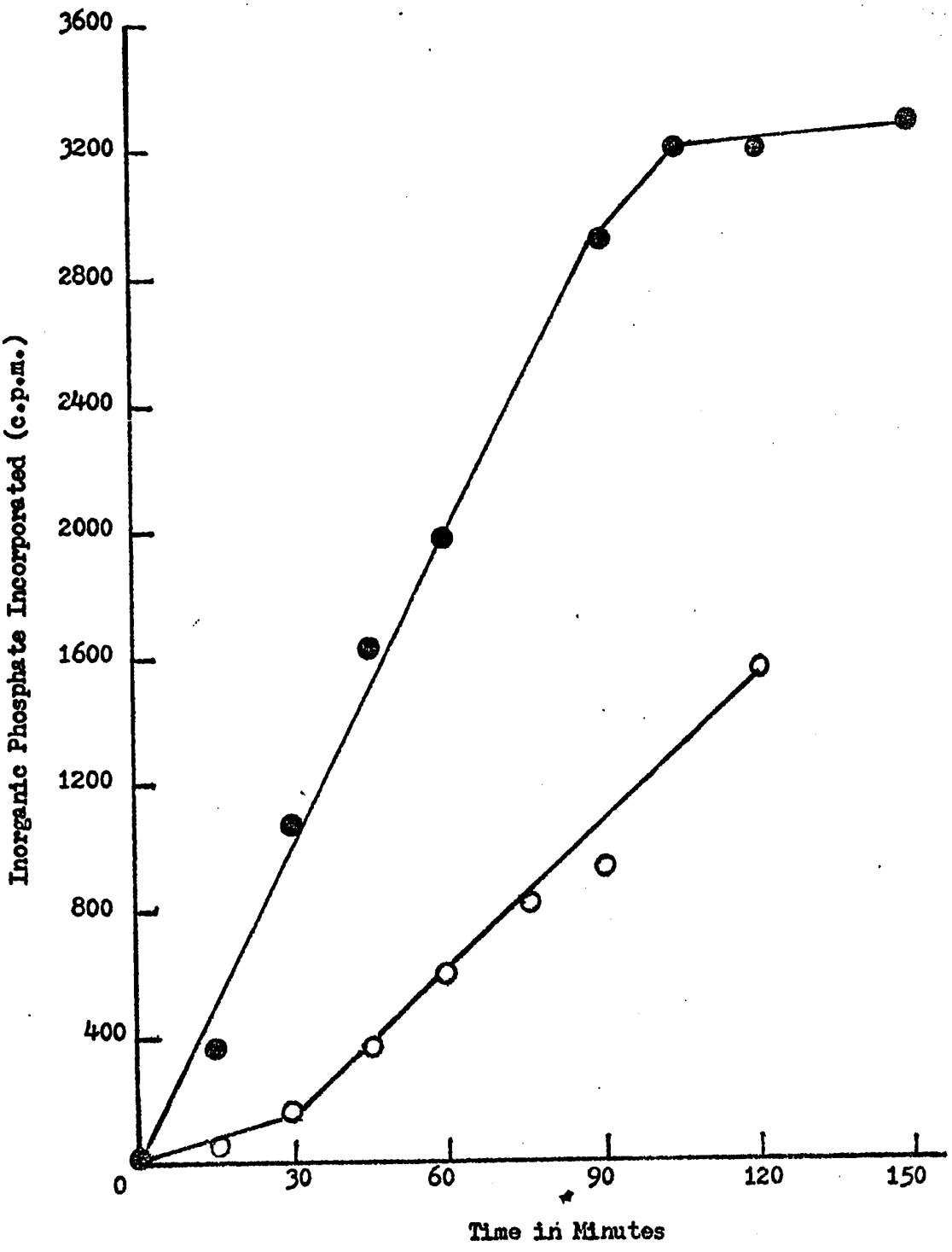


Figure 20: Time-course of poly A phosphorolysis by guinea-pig and rat liver nuclear polynucleotide phosphorylase. Polynucleotide phosphorylase activity was measured in the standard assay conditions. Guinea-pig,(●); rat,(○).

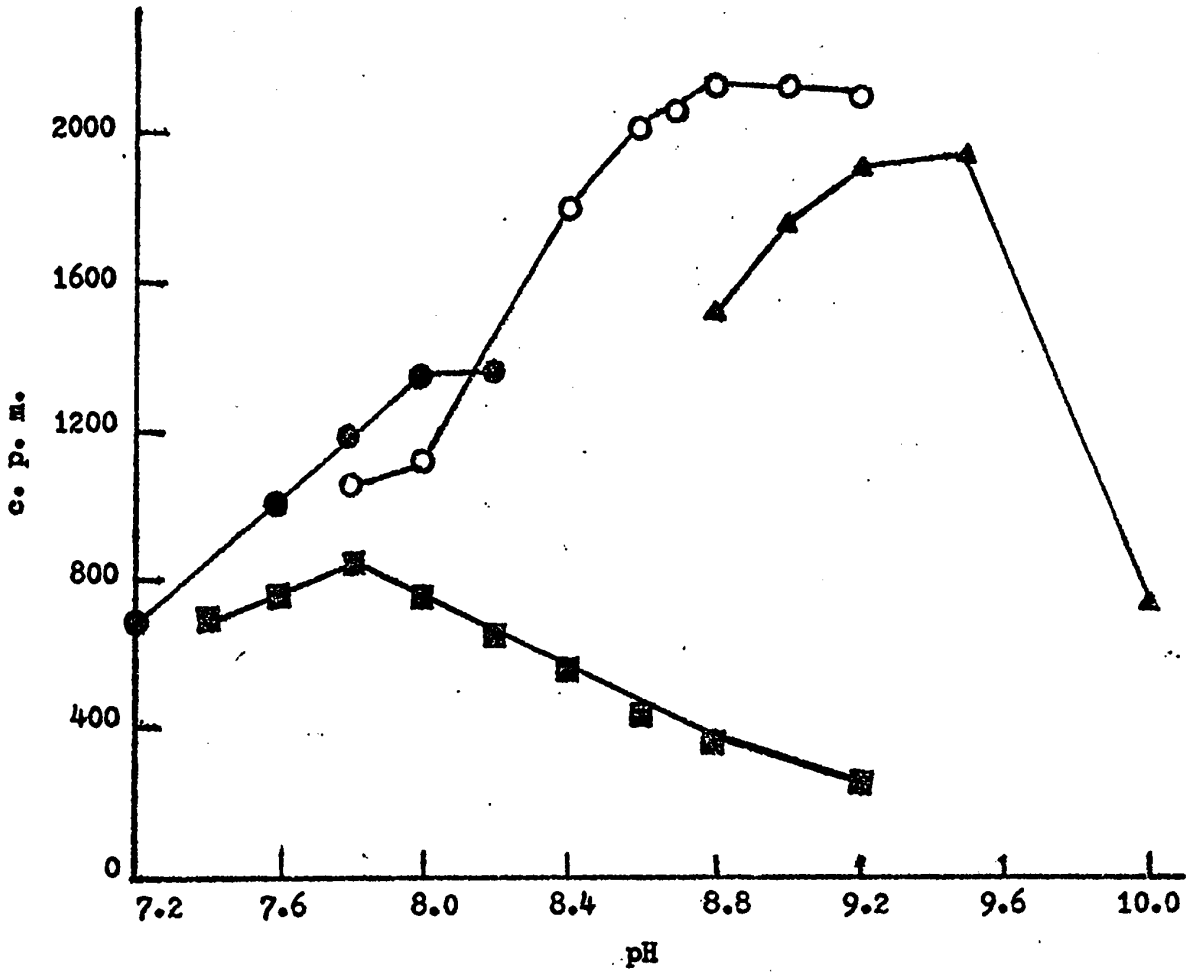


Figure 21: Dependence of phosphorolysis activity of guinea-pig and rat liver nuclear polynucleotide phosphorylase on pH. Enzymic activity was determined in the standard poly A phosphorolysis conditions with 0.1 M buffers of the indicated pH and composition. Guinea-pig liver nuclear enzyme;(●) imidazole buffers; (○) tris-HCl buffer; (▲) glycine-NaOH buffer. Rat liver nuclear enzyme: (■) tris-HCl buffer.

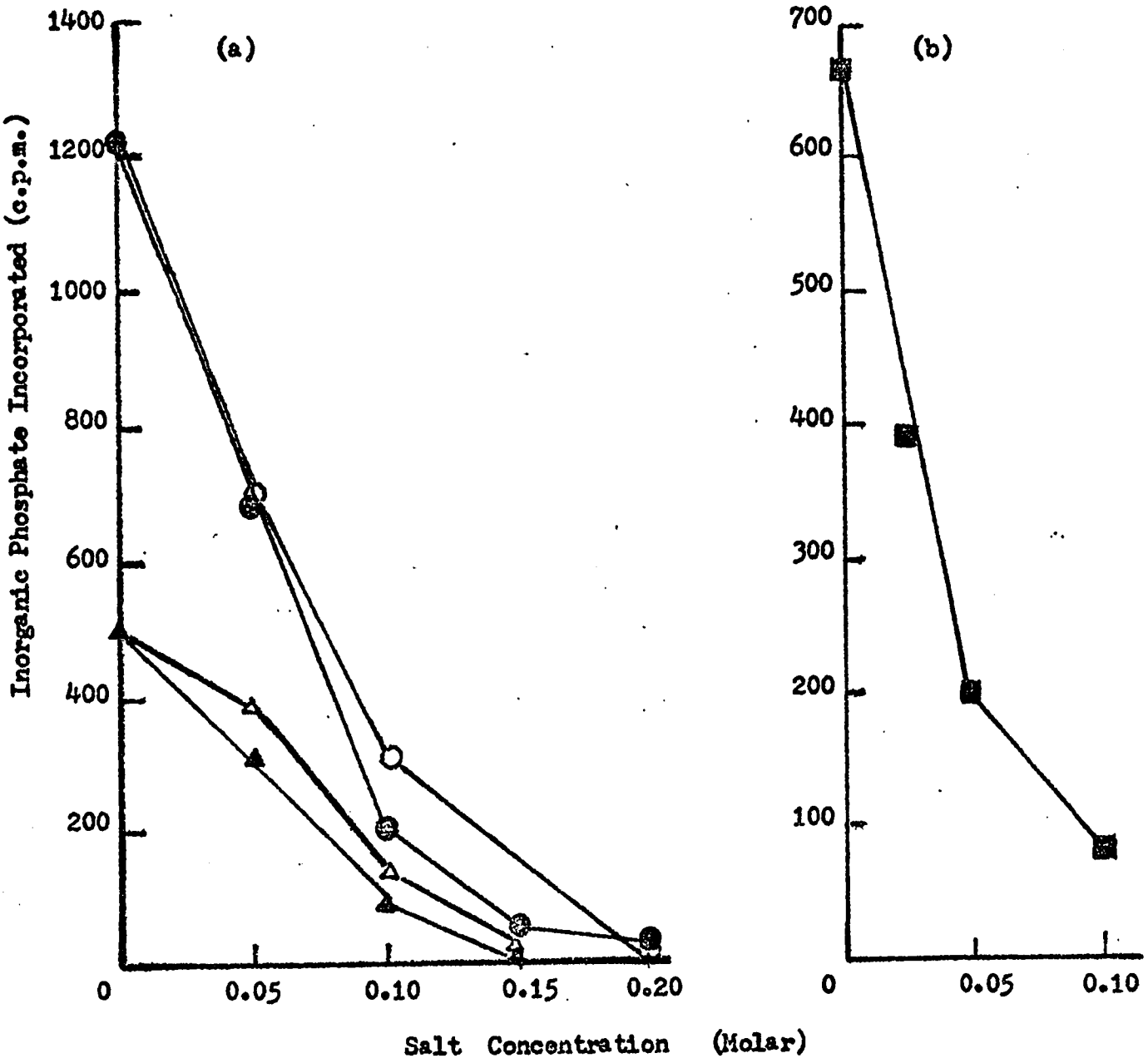


Figure 22: Effect of salt on the phosphorolysis activity of guinea-pig and rat liver nuclear polynucleotide phosphorylase. Enzymic activity was measured by the standard assay at the indicated salt concentrations. (a) Guinea-pig liver nuclear enzymes: (▲), NaCl, saline fraction; (△), KCl, saline fraction; (●), NaCl, sucrose fraction; (○), KCl, sucrose fraction. (b) Rat liver nuclear enzyme: (■), NaCl, sucrose fraction.

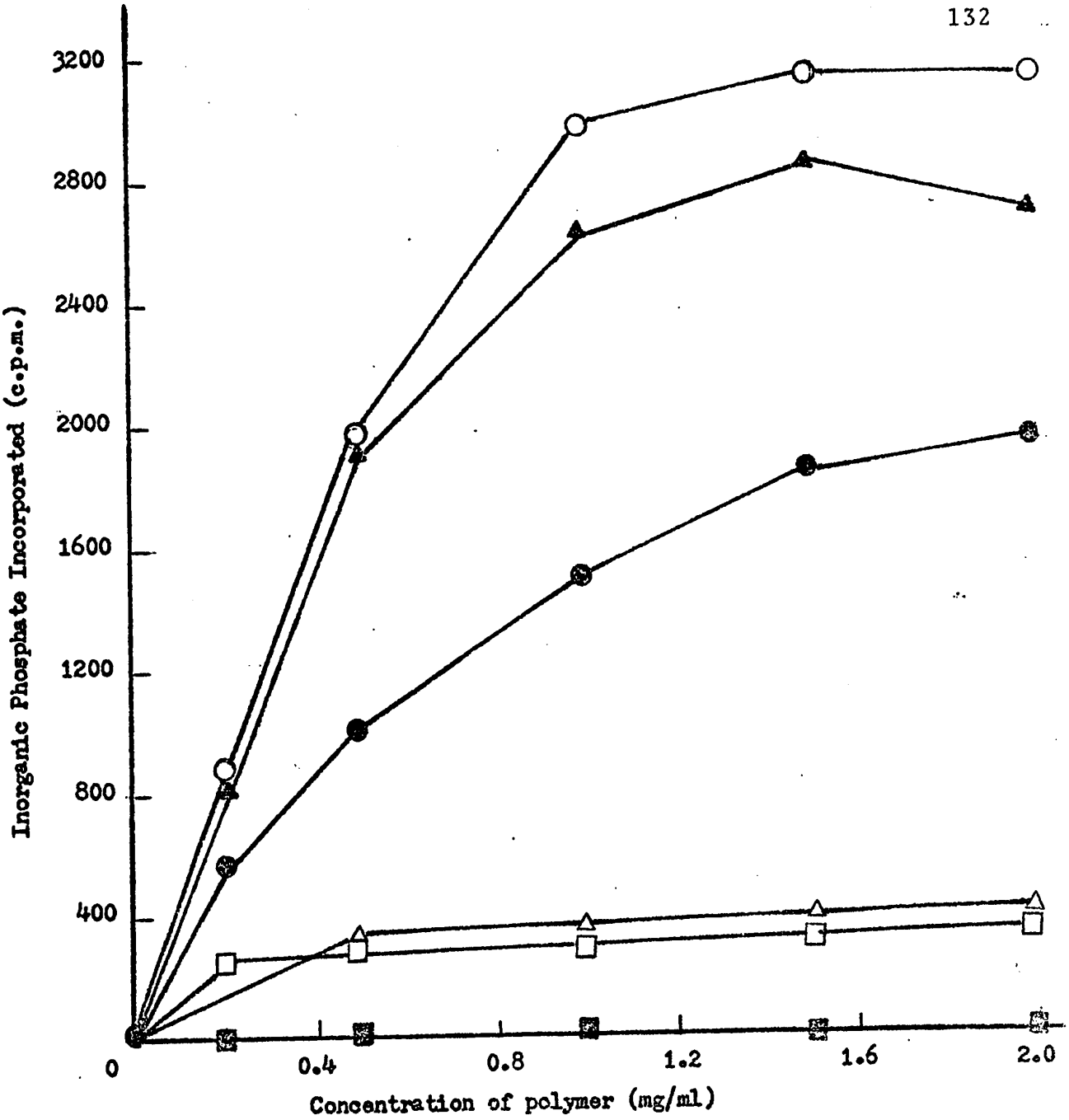


Figure 23: Substrate specificity of guinea-pig liver polynucleotide phosphorylase. The phosphorolysis activity was measured in the standard assay with the indicated concentrations of polyribonucleotide. (○), poly U; (▲), poly C; (●), poly A; (■), poly G; (△), wheat germ rRNA; (□), yeast tRNA.

rylase (Babinet et al., 1965). The Mg^{2+} optimum for the sucrose and saline fraction was 4mM (Fig. 18(a)). The rat liver nuclear polynucleotide phosphorylase also required divalent cation for activity, with a Mg^{2+} optimum of 6mM (Fig. 18(b)).

Fig. 19 shows the enzyme concentration curve for both guinea-pig and rat liver nuclear enzyme (sucrose fraction). The enzyme concentration curves were linear except at low enzyme concentrations. The time course of reaction for the guinea-pig liver nuclear enzyme was linear between 30 to 90 minutes and between 30 to 120 minutes for the rat liver nuclear enzyme (Fig. 20).

The pH optimum for the guinea-pig liver nuclear enzyme varies with buffers. The maximum activity was observed between pH 8.6 and 9.2 in tris-HCl buffer (Fig. 21). The rat liver nuclear enzyme had a pH optimum at 7.8 with tris-HCl buffer. The effects of buffers on the activity of the latter enzyme were not studied.

The sucrose and saline fractions of guinea-pig liver nuclear enzyme were inhibited markedly by sodium and potassium chloride (Fig. 22(a)) and were inactive when the salt concentration in the reaction mixture was 0.2M or higher. The rat liver nuclear enzyme was inhibited in the same manner by sodium chloride (Fig. 22(b)).

Fig. 23 shows the substrate specificity of the sucrose fraction of the guinea-pig liver nuclear enzyme. It catalysed phosphorolysis of poly U, poly C and poly A; was less active towards t-RNA or high molecular weight RNA, and had no activity with poly G or DNA.

TABLE 18

Effect of KCl and NaCl on ADP-P_i and ATP-P_i exchange activities of the particulate guinea-pig liver enzyme

Exchange activities were assayed as described in the Methods section at the indicated salt concentration.

Salt	Concn. (mM)	Exchange activity		Poly A Phosphorolysis
		ADP-P_i	ATP-P_i	
		c.p.m. incorporated		
None	-	198	166	2009
KCl	5	260	198	-
KCl	10	271	221	1515
NaCl	5	226	181	-
NaCl	10	269	188	1512
KCl + NaCl	5 + 5	394	225	1696

TABLE 19

Effect of ApA on phosphorolysis and exchange reactions of guinea-pig nuclear polynucleotide phosphorylase

Poly A phosphorolysis, ATP/P_i and ADP/P_i exchange reactions were measured in the standard conditions described for guinea-pig nuclear enzyme, with purified sucrose fraction. The concentration of ApA, when present, was 3.2 mM.

Reactions	^{32}P-P_i incorporated (c.p.m.)	
	- ApA	+ ApA
poly A phosphorolysis	2482	2647
ATP/P_i exchange	146	101
ADP/P_i exchange	251	216

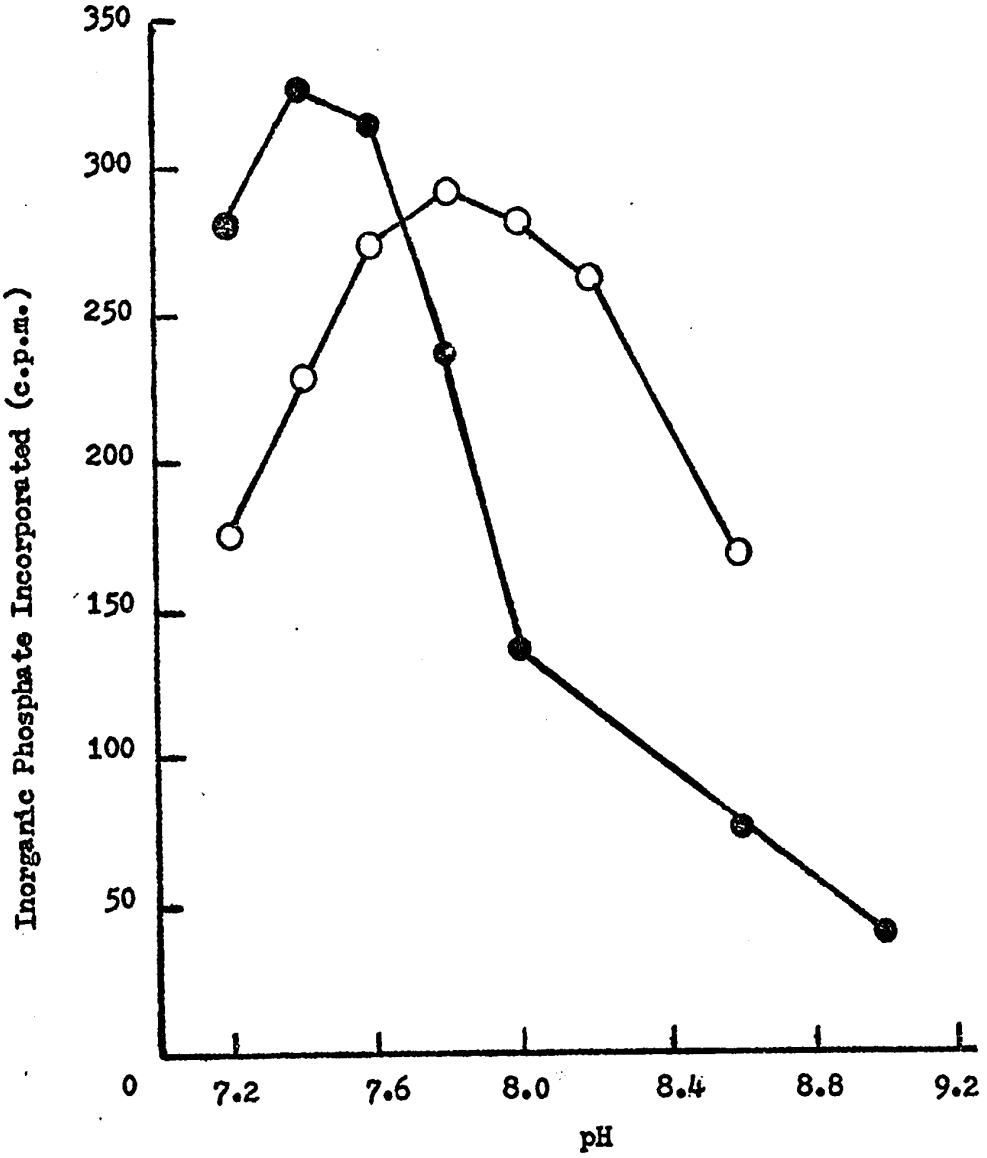


Figure 24: Effect of pH on ADP/Pi and ATP/Pi exchange reactions associated with guinea-pig nuclear polynucleotide phosphorylase. The ADP/Pi and ATP/Pi exchange activities were assayed in the standard assay conditions in the presence of 10 mM KCl as activator, with 0.1 M imidazole-HCl (pH 7.2-8.2) and 0.1 M tris-HCl buffer (pH 8.6-9.0): (●), ATP/Pi exchange; (○), ADP/Pi exchange.

TABLE 20

The change in ATP/P_i and ADP/P_i exchange activity associated with guinea-pig nuclear polynucleotide phosphorylase during purification

The changes in ATP/P_i and ADP/P_i exchange activities were followed during the purification of the sucrose extracts of guinea-pig liver nuclei. The ATP/P_i and ADP/P_i exchange activities were assayed in the standard assay conditions described for these activities in nuclear enzyme (see Methods).

Step	ATP/P_i exchange			ADP/P_i exchange		
	Units	Protein (mg)	Sp. activity (units/mg)	Units	Protein (mg)	Sp. activity (units/mg)
1. Crude extract	30600	194	158	30702	194	158
2. pH 5.7	17120	66	261	2164	66	330
3. DEAE-cellulose 0.3M-NaCl eluate	385	5.6	69	530	5.6	95

Initially, the guinea-pig liver nuclear enzyme was extracted in the absence of reducing agents, but subsequent purification always led to a great loss of activity. The inclusion of 2-mercaptoethanol in the buffers stabilized the enzyme. The effect of 2-mercaptoethanol on the enzyme was therefore studied. When the purified particulate enzyme was dialysed overnight against buffer to remove 2-mercaptoethanol, only 40% of its activity could be recovered.

The partially purified guinea-pig liver nuclear enzyme was also associated with low ATP/P_i and ADP/P_i exchange activities. Both exchange activities were stimulated by low concentrations of potassium and sodium chloride, whereas the phosphorolytic activity was inhibited by the same concentrations of salts (Table 18). The ATP/P_i and ADP/P_i exchanges were inhibited 31% and 14% respectively, by ApA (3.2mM) whereas the phosphorolytic activity was not affected or slightly stimulated by the same concentration of the dinucleotide (Table 19). The pH optima for the ATP/P_i and ADP/P_i exchange activities were 7.4 and 7.8 respectively when assayed in the presence of 10mM potassium chloride as activators (Fig. 24). The activator was included in these assays because of the very low exchange activity of the preparation. The pH optima of both exchange activities were considerably lower than that for the phosphorolytic activity and were not affected by the buffer used (Fig. 24 and 21). The ATP/P_i and ADP/P_i exchange activities fell considerably during purification (Table 20) of the polynucleotide phosphorylase (cf. Table 13). In contrast to the phosphorolytic activity, both exchange activities were

TABLE 21

Comparison of the substrate specificity for exchange and phosphorolysis of the particulate guinea-pig liver nuclear enzyme

Exchange and phosphorolytic activity of the sucrose fraction were determined as described in the Methods section.

Reaction	Nucleotide or polymer base (N)			
	A	C	G	U
	(c.p.m. incorporated)			
NTP/P_i exchange	284	40	99	19
NDP/P_i exchange	289	43	23	26
Polynucleotide phosphorolysis	2091	2641	0	3253

specific for the adenine nucleotides and have little activities on other nucleotides (Table 21). All these observations suggested that the exchange activities and the phosphorolytic activity were due to different enzymes.

It has so far been impossible to detect any nucleoside diphosphate polymerization activity in the guinea pig liver nuclear polynucleotide phosphorylase preparations over a wide range of pH and divalent cation concentrations, both with and without oligonucleotide primers, salts and polylysines (see p.6-13 for discussion).

(ii) Is the extracted nuclear polynucleotide phosphorylase of nuclear origin ?

Rat liver instead of guinea-pig liver was used for the studies reported below for the following reasons: (a) the properties of the rat liver mitochondrial polynucleotide phosphorylase have been studied in detail (see section II B); (b) the purified rat liver nuclear enzyme contained cardiolipin, characteristic of mitochondrial inner membrane; (c) most studies of animal metabolism have been performed with rats.

Polynucleotide phosphorylase was isolated and partially purified from guinea-pig liver and rat liver nuclei prepared by the 2.2M-sucrose procedure as was discussed in the previous section. However, no activity could be detected when intact or sonicated nuclei were assayed, even after 3 hour incubation. This is most probably due to the high phosphatase activities detected in whole nuclei. When 1mM ^{14}C-ADP was incubated

with the same amount of sonicated nuclei as used for the detection of polynucleotide phosphorylase activity, 62% of the ^{14}C-ADP was converted to ^{14}C-AMP by phosphatases and 12% to ^{14}C-ATP by adenylate kinase.

Since the ^{32}P-orthophosphate incorporated during phosphorolysis is present in the β-phosphate group of ADP and this group is lost during hydrolysis of the nucleoside diphosphate to the monophosphate, high concentrations of active phosphatases can prevent detection of polynucleotide phosphorylase activity. However, if the polynucleotide phosphorylase activity is high compared with the phosphatase activity, its activity should be detectable. This is the case with the mitochondrial inner membrane preparation, where the phosphatases degraded a large proportion of the added ^{14}C-ADP (1mM) to ^{14}C-AMP (Table 1, p. 68).

The ^{14}C-ATP formed was produced by adenylate kinase which catalysed the reaction.

$$2ADP^{*} \rightleftharpoons ATP^{**} + AMP$$

In this case, no labelled phosphate is lost and the doubly labelled ATP is charcoal adsorbable. The presence of adenylate kinase or similar enzymes will, therefore, not interfere with the phosphorolysis assay.

The nuclear pellets obtained by the 2.2M-sucrose procedure (Method xvi) were contaminated by haemoglobin. The Whittle et al. (1968) modification of the Blobel and Potter procedure (Method xiv) gave white nuclear pellets, but they contained no extractable or detectable polynucleotide phosphorylase. Nuclear membrane prepared from these nuclei also contained no detectable polynucleo-

tide phosphorylase activity. Since only a small sample of tissues (maximum, 15g) could be handled by this procedure, a large-scale modification of the Blobel and Potter procedure was developed (Method xv) that could be used with 90g liver (the amount used in the 2.2M-sucrose method). The nuclei isolated by this procedure also lacked polynucleotide phosphorylase.

To eliminate the possibility that the polynucleotide phosphorylase extracted from the 2.2M-sucrose nuclei were from contaminating blood cells, the livers were perfused with 0.25M-sucrose in tris-ME buffer and the blood was collected and assayed for polynucleotide phosphorylase activity. There was no detectable polynucleotide phosphorylase in the blood and the nuclei prepared from the perfused livers by the 2.2M-sucrose procedure contained the same amount of extractable polynucleotide phosphorylase as those from non-perfused livers. Thus, the polynucleotide phosphorylase obtained from nuclei prepared by the 2.2M-sucrose procedure was not due to blood contamination.

Since nuclei isolated from either guinea-pig liver or rat liver by the 2.2M-sucrose procedure (Method xvi) always yielded polynucleotide phosphorylase, whereas those prepared by the Blobel & Potter procedures (Methods xiv and xv) were free from the enzyme, a detailed study was made in an attempt to establish if this activity were genuinely nuclear or due to mitochondrial contamination.

It was first determined that the nuclei isolated directly by homogenization and centrifugation in 2.2M-sucrose (Method xvi) contained 1-3% of the total liver mitochondria, in agreement with the level of mitochondrial contamination previously reported

TABLE 22

Mitochondrial contamination of nuclei prepared by the 2.2M sucrose procedure

^{32}P-labelled-mitochondria procedure

^{32}P-labelled mitochondria were prepared as described in Method IX. The labelled mitochondria were suspended in 5 ml 0.25M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (sucrose-tris-ME buffer) and added to 6 rat livers in 2.2M sucrose-1mM-2-mercaptoethanol-10mM tris-HCl, pH 7.4 (4 ml/g liver). The livers were homogenized and nuclei isolated as described by method xvi. The nuclear pellet was suspended in 42 ml sucrose-tris-ME buffer and 0.2 ml samples were taken for scintillation counting. Polynucleotide phosphorylase were extracted and purified from the rest of the nuclei (Method xviii).

Organelle	Total ratioactivity (d.p.m.)	Percent radioactivity recovered	Percent contamination (corrected for 9 rats)
Mitochondria	3.41×10^6	-	-
Isolated nuclei	3.0×10^4	0.88	2.64
Nuclear residue after enzyme extraction	1.41×10^4	0.41	-
Purified particulate enzyme	93	0.003	-

TABLE 23

Mitochondrial contamination of nuclear pellet prepared by the 2.2M-sucrose procedure

Cytochrome oxidase procedure

Six rat livers were homogenized in 2.2M-sucrose-10mM-tris-HCl, pH 7.4 as described in method xvi. 0.1 ml sample was taken and diluted with 2 vol. of 10mM-tris-HCl buffer and used for cytochrome oxidase determination. The rest of the homogenate was processed as described in method xvi for the isolation of nuclei. The final nuclear pellets were resuspended in 0.25M-sucrose-10mM-tris-HCl buffer, pH 7.4 (5.4 ml). Samples were taken for cytochrome oxidase assay.

Fractions	Cytochrome oxidase (units)	Mitochondrial contamination (%)
crude homogenate	2074.3	-
nuclear pellet	29.6	1.42

TABLE 24

A comparison of the polynucleotide phosphorylase and cytochrome oxidase activities of rat liver nuclei obtained by different procedures

Nuclei were isolated from rat liver (90 g) by either (i) 2.2M-sucrose (Method xvi) or (ii) 0.25M/2.2M-sucrose (Method xvii), using buffers that lacked 2-mercaptoethanol. DNA, cytochrome oxidase (cyt. oxidase) and polynucleotide phosphorylase (PNPase) were determined as described in the methods section.

Expt.	Nuclei	cyt. oxidase (units)	PNPase (units)	DNA	PNPase / DNA	cyt. oxidase / DNA
1.	2.2M-sucrose	29.6	2284	5.1	438	5.80
2.	0.25/2.2M-sucrose	3.92	684	3.7	184	1.06

$$\frac{\text{PNPase/DNA, Expt. 1}}{\text{PNPase/DNA, Expt. 2}} = 2.4$$

$$\frac{\text{cyt. oxidase/DNA, Expt. 1}}{\text{cyt. oxidase/DNA, Expt. 2}} = 5.5$$

(Berezney et al., 1970) in nuclei isolated by a modified 2.2M-sucrose procedure (similar to Method xvii). Two methods were used. In the first, radioactive rat liver mitochondria were prepared after being labelled in vivo by the procedure of McMurray & Dawson (1969) (Method ix). They were added to unlabelled rat liver from which nuclei were then isolated as usual (Method xvi) in 2.2M-sucrose. After correction for dilution of the labelled mitochondria by the organelles in the unlabelled liver, about 3% of the total liver mitochondria were found to be associated with the nuclei (Table 22). In separate experiments, the separation of mitochondria and nuclei was followed by determination of the cytochrome oxidase activity in the total crude rat liver homogenate in 2.2M-sucrose and in the nuclei obtained after high-speed centrifugation of the latter using buffer without 2-mercaptoethanol (Method xvi). By this method, it was found that 1-2% of the cytochrome oxidase activity remained associated with the crude nuclear fraction (Table 23), in good agreement with the level of contamination found independently with radioactive mitochondria.

Table 24 describes two experiments in which the cytochrome oxidase and polynucleotide phosphorylase activity/mg of DNA present in nuclei isolated by two methods in the absence of 2-mercaptoethanol were compared. It can be seen that the nuclei with the least mitochondrial contamination (i.e., with least cytochrome oxidase activity) also contained the least polynucleotide phosphorylase. Further, when the nuclei isolated in 2.2M-sucrose alone were purified by the resuspension in the high sucrose-buffer and recentrifu-

TABLE 25

The similarity of rat liver nuclei and mitochondrial inner membrane polynucleotide phosphorylase

Properties	Polynucleotide phosphorylase	
	Nuclear	Mitochondrial
1. physical state	solid (membrane bound)	solid (membrane bound)
2. pH optimum	7.8	7.8
3. Mg^{2+} optimum	6mM	6mM
4. NaCl inhibition (0.1 M)	88%	86%

gation, the purified nuclei contained no polynucleotide phosphorylase: however, the recovery of nuclei by this method was very poor. Nevertheless, the result agreed with that obtained with nuclei isolated in good yield by the modified, large-scale Blobel and Potter procedure (Method xv) which also lacked polynucleotide phosphorylase activity whether the assays were performed on intact nuclei or extracts prepared by our usual method, or on isolated nuclear membranes (Method xiv). Finally, the enzyme isolated from rat liver nuclei by the procedure used (Methods xvi and xix) to prepare guinea-pig liver polynucleotide phosphorylase not only resembled the rat liver mitochondrial polynucleotide phosphorylase very closely (Table 25) but contained cardiolipin, which is considered to be present exclusively in mitochondria (Getz, 1970).

Thus, the evidence presented above is generally consistent with rat liver polynucleotide phosphorylase and, by extension, guinea-pig liver polynucleotide phosphorylase (Fitt and See, 1970; See and Fitt, 1970), being a mitochondrial enzyme found in crude nuclei only as a contaminant and may be summarized as follows: (i) highly purified nuclei contain no polynucleotide phosphorylase; (ii) the amount of polynucleotide phosphorylase present in nuclei decreases as the purity of the latter increases and the change roughly parallels the change in cytochrome oxidase activity in the preparation; (iii) the purified "nuclear" enzyme from rat liver contains cardiolipin, typical of the mitochondria, and has enzymic properties apparently identical with those of the mitochondrial enzyme.

TABLE 26

Comparative isolations of rat liver polynucleotide phosphorylase from mitochondria and nuclei by osmotic shock

Crude nuclei were isolated from rat liver (90 g) by the 2.2M-sucrose procedure (Method xvi). Polynucleotide phosphorylase was extracted from the nuclei with 0.25M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 (sucrose-tris-buffer) and purified by pH 5.6 precipitation and DEAE-cellulose chromatography (method xviii).

Mitochondria (from 90 g liver) were suspended in 2.2M-sucrose-1mM-2-mercaptoethanol-10mM-tris-HCl, pH 7.4 and polynucleotide phosphorylase was extracted by osmotic shock (method xx), by diluting the 2.2M-sucrose to 0.25 M-sucrose with 1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4 followed by resuspension in a Dounce homogenizer. The extracted polynucleotide phosphorylase was purified by pH 5.6 precipitation and DEAE-cellulose chromatography (method xviii).

Treatment	phosphorolysis activity (units)		
	Mitochondria		Nuclei
	Expt. 1	Expt. 2	
1. Whole mitochondria	9108	8080	-
2. (i) osmotic shock:			
(a) crude extract	5260	4460	2066
(b) 3% of (a)	158	134	-
(ii) pH 5.6 precipitation	3680	-	3150
(iii) DEAE-cellulose column			
(a) total recovery	208	-	350
(b) 3% of (a)	6.2	-	-

However, the possibility still remains that the enzyme is associated with nuclei as well as with mitochondria. The results in Table 26 show that, even if all the polynucleotide phosphorylase present in mitochondria could be extracted by the osmotic shock procedure used (Method xx) for preparation of the crude "nuclear" enzyme, 25% of the mitochondria of rat liver would have to be present in the crude nuclei to account for the enzyme activity extracted from the latter. However, it has been shown (see above) that a maximum of 3% of the mitochondria contaminate the nuclei. Further, extraction of isolated mitochondria by the procedure used for nuclei yields (Table 26) only 50-60% of the total mitochondrial polynucleotide phosphorylase and it could then only be purified with much greater losses than the enzyme from the crude nuclei, so that only 1.8-3.5% of the activity of the purified "nuclear" enzyme could arise from contaminating mitochondria. Finally, if the isolated mitochondria were first homogenized in 2.2M-sucrose, in the conditions used for preparation of the crude homogenate prior to isolation of the nuclei by the standard procedure (Method xvi), and then submitted to osmotic shock by dilution of the medium to 0.25M-sucrose followed by homogenization in a Dounce homogenizer (Method xx), the yield of polynucleotide phosphorylase was only 20% of that obtained by omission of the first homogenization step before dilution of the suspension. These results are difficult to reconcile with an exclusively mitochondrial location for rat liver polynucleotide phosphorylase and suggest that it may in fact be present in the nucleus. This implies that the methods necessary for the isolation of highly-purified nuclei may lead to a loss of polynucleotide phosphorylase.

TABLE 27

Intracellular localization of rat liver polynucleotide phosphorylase

Sub-cellular fractions were isolated as described in the Methods section and their polynucleotide phosphorylase activity measured by the standard phosphorolysis assay. The activity in the nuclei was determined using extracts prepared by method xviii.

Fraction	Activity	
	units/mg protein	units/g (wet wt.) tissue
1. Mitochondria	120-191	335-602
2. Nuclei		
(i) 2.2M sucrose nuclei	10-32	15-27
(ii) Blobel and Potter nuclei	none detectable	
3. Glycogen particles Microsomes Plasma membrane Soluble fraction	none detectable	

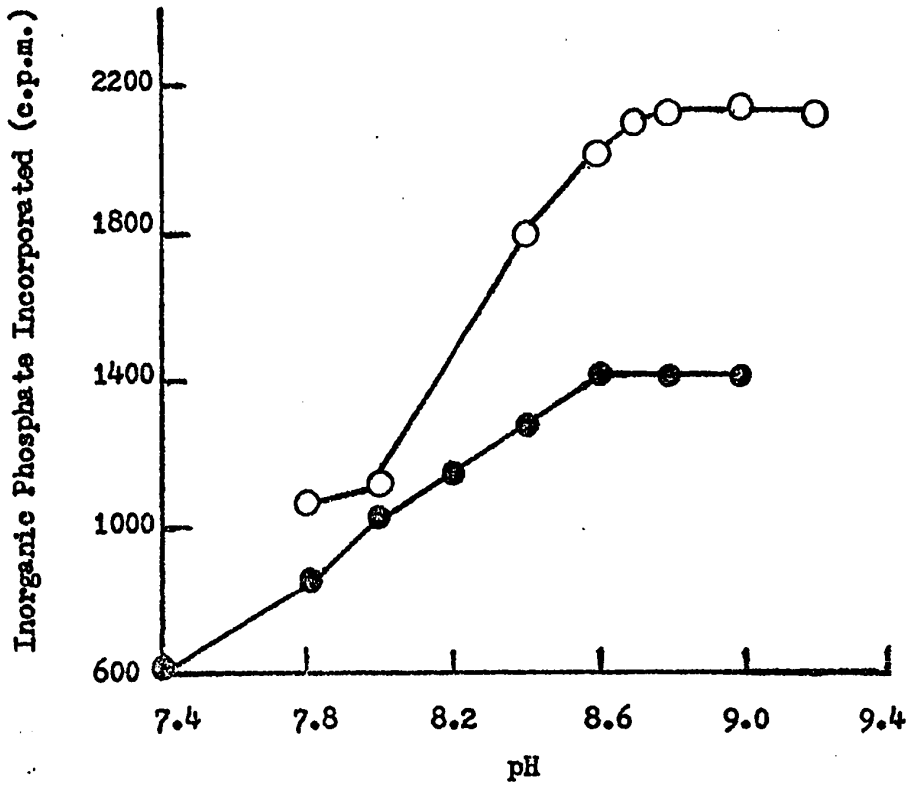


Figure 25: Effect of pH on guinea-pig liver mitochondrial and partially purified nuclear polynucleotide phosphoylase. Mitochondria were isolated as described for rat liver mitochondria and the guinea-pig liver nuclear enzyme was extracted and purified as described in section II c. The nuclei were prepared by the 2.2 M-sucrose procedure (Method xvi). The poly A phosphorolysis was measured in the standard conditions described in the method section. (●), mitochondria; (○) partially purified nuclear enzyme assayed in the absence of Triton X-100.

TABLE 28

Distribution of mitochondrial polynucleotide phosphorylase in rat tissues

Mitochondria were isolated from the indicated rat tissues as described in the Methods and their polynucleotide phosphorylase activity determined by the standard phosphorolysis assay, in the presence of 0.1% (v/v) Triton X-100.

Tissues	Polynucleotide phosphorylase activity	
	units/g tissue (wet wt.)	units/mg protein
Heart	526	78
Kidney	1773	200
Liver	602	191
Muscle (thigh)	38	28
Spleen	248	18

3. Localization and distribution of animal polynucleotide phosphorylase

(i) Intracellular distribution of rat liver polynucleotide phosphorylase

Table 27 shows the distribution of poly A phosphorolysis in subcellular fractions of rat liver isolated as described in the Methods section. In all cases, the activities were assayed in the conditions found optimum for the membrane-bound rat liver mitochondrial and nuclear enzyme, pH 7.8 (Figs. 3 and 21) and also at pH 8.8, at which the guinea-pig liver enzymes are most active (Figs. 21 and 25). Polynucleotide phosphorylase activity could only be detected unequivocally in the mitochondria. The presence of polynucleotide phosphorylase in nuclei is uncertain and had been discussed in detail in section II D. The glycogen particles, microsomal fraction, plasma membrane and the soluble fraction of the liver cells contained no detectable polynucleotide phosphorylase under the conditions used for enzyme assay (Table 27).

(ii) Distribution of mitochondrial polynucleotide phosphorylase in rat tissues

Table 28 shows the results of a study of the level of polynucleotide phosphorylase activity in mitochondria isolated from several tissues of the rat. Assays were performed at both pH 7.8 and 8.8, and in all cases the phosphorolytic activity was higher at pH 7.8. The specific activity of kidney and liver mitochondrial polynucleotide phosphorylase was similar, but kidney gave the

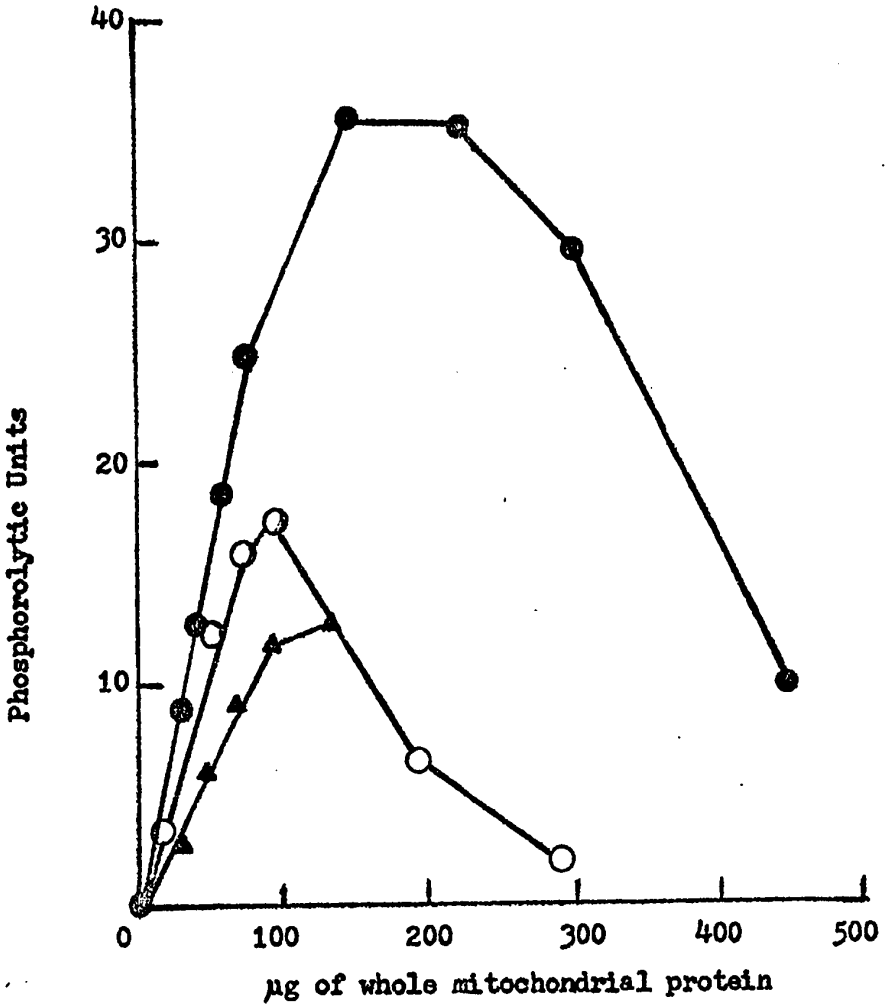


Figure 26: Enzyme-saturation curve for whole mitochondrial polynucleotide phosphorylase. Polynucleotide phosphorylase activity was measured by the standard poly A phosphorolysis assay in the presence of 0.1% (v/v) Triton X-100. Mitochondria from different animal tissues were examined: (●), guinea-pig liver; (○), rat kidney; (▲) bovine liver.

highest yield of units/mg (wet wt.) of tissue. I have not studied the effect on the age or nutritional state of the polynucleotide phosphorylase and phosphatase(s) levels in different tissues, so too much significance should not be attached to the apparent relative abundance of the enzyme. However, it is clear that mitochondrial polynucleotide phosphorylase is present in significant amounts in all the tissues tested.

It should be emphasized that earlier studies (Table 4) have shown that 89% of the polynucleotide phosphorylase activity detectable in intact mitochondria in the presence of Triton X-100 is recoverable in isolated inner membranes and that nucleoside diphosphates are the main products of the phosphorolysis of polyribonucleotides (Fig. 1). The activity of whole mitochondria in the assay is thus a true measure of their polynucleotide phosphorylase content. In the presence of Triton X-100 as used in the standard assay, level of incorporation of radioactivity into controls with whole or sonicated mitochondria, but in the absence of polyribonucleotides, were similar to that in the controls with polyribonucleotides, but without mitochondria (Table 2).

The enzyme saturation curve for whole mitochondrial polynucleotide phosphorylase (Fig. 26) was similar to that shown for the rat liver inner membrane enzyme (Fig. 7) and was bell-shaped (Fig. 26), so that the measured activity fell above 90 µg protein in the assay.

(iii) Occurrence of mitochondrial polynucleotide phosphorylase in different animal species

A comparative study was also made of the occurrence of mito-

TABLE 29

Occurrence of mitochondrial polynucleotide phosphorylase in various animal species

Mitochondria were isolated from the tissues indicated as described in the Methods and their polynucleotide phosphorylase activity determined by the standard phosphorolysis assay in the presence of 0.1% (v/v) Triton X-100.

Tissues	Polynucleotide phosphorylase activity	
	units/g tissue (wet wt.)	units/mg protein
Bovine liver	1215	176
Gold-fish muscle	78	37
Guinea-pig liver	2964	309
Rabbit liver	721	310
Rat liver	602	191
Rooster liver	1715	307

chondrial polynucleotide phosphorylase in bovine, guinea-pig, rabbit, rat and rooster livers and in goldfish muscle. The results in Table 29 show that the enzyme was present in all cases and is therefore widespread amongst animal species. The assays were performed at both pH 7.8 and 8.8, which had been found in detailed studies to be the optima for the rat (Fig. 3) and guinea-pig liver mitochondrial enzymes (Fig. 25), respectively. The enzymes from all the other species tested so far resembled the guinea-pig mitochondrial polynucleotide phosphorylase rather than the rat enzyme and were more active at pH 8.8. However, it should be noted that the partially purified rat mitochondrial polynucleotide phosphorylase has a pH optimum of 8.2 (Fig. 3) and that at least part of the difference between the crude and purified enzyme is due to the high phosphatase activity in the crude rat liver mitochondrial inner membrane preparations (section II C).

III. A. Determination of inorganic phosphate in the presence of Triton X-100

Introduction

Many procedures have been developed for the colorimetric determination of inorganic orthophosphate (see review by Lindberg and Ernster, 1956). Most of these procedures depend on the formation of phosphomolybdic acid complex and its reduction to molybdenum blue.

During the studies of the polynucleotide phosphorylase of rat liver mitochondrial membranes (section II C), it was necessary to assay phosphatase, pyrophosphatase and nucleoside diphosphatase activities in the presence of Triton X-100 used to solubilize the membrane-bound polynucleotide phosphorylase. The assays depended on the determination of orthophosphate and it is known (Wattiaux and De Duve, 1956; Eibl and Lands, 1969), and I have confirmed, that Triton X-100 interferes with the Fiske and SubbaRow method (Fiske and SubbaRow, 1925; Gomori, 1942) by precipitating the phosphomolybdate complex. Eibl and Lands (1969) found that the increase in turbidity in the absence of a reducing agent was proportional to orthophosphate concentration and could be made the basis of a sensitive phosphate assay, but their method was not convenient owing to its low upper limit of proportionality. Roufogalis (1971) has recently published a modification of the Fiske and SubbaRow assay that can be used in the presence of Triton X-100 and certain other interfering substances, which are removed by prior precipitation with silicotungstic acid.

It was found that the precipitated phosphomolybdate complex redissolves if the concentration of Triton X-100 is sufficiently high and use was made of this observation to develop two rapid methods for the accurate estimation of orthophosphate in the presence of the detergent. They are modifications of the Gomori modification of the Fiske and SubbaRow procedure (Gomori, 1942) and of the Lowry-Lopez method (Lowry and Lopez, 1946), respectively.

Materials

Triton X-100 was purchased from Canadian Laboratory Supplies Ltd., Montreal, P.Q. and p-methylaminophenol sulphate (Elon) from Eastman Kodak Co., Rochester, N.Y. All other reagents were obtained from Fisher Scientific Co., Ottawa, Ont.

Methods

Method Ia. The following stock reagents are required: (A) 2% (v/v) Triton X-100 in 1M glycine (when fresh this reagent is slightly turbid, but this does not affect the assay); (B) 1% (w/v) ammonium molybdate tetrahydrate in 0.05N sulfuric acid (this reagent may be used for at least a month provided it is stored in the dark in plastic, not glass, bottles); (C) sodium metabisulphite (15 gm) and p-methylaminophenol sulphate (5 gm) in water (500 ml).

Water (1 ml) and solutions A, B and C (1 ml of each) are added in the order indicated to 1 ml of the test sample containing 3% (w/v) perchloric acid and 0.05-1.0 µmole of orthophosphate (final volume: 5 ml). The final pH of the solutions is 2.5-2.6. The mixtures are allowed to stand at room temperature for 30 min and their extinction at 650 nm is determined spectrophotometrically

using 1 cm pathlength cells. The blank is a similar mixture lacking orthophosphate.

For enzyme assays, the reactions are stopped by the addition of an equal volume of 6% (w/v) perchloric acid. Precipitated protein (including carrier, if required) is removed by centrifugation and 1 ml of the supernatant is used for phosphate determination.

Method Ib. This is a modification of Method Ia for use with samples containing sucrose. The order of addition of the reagents is then A, C, B. In all other respects, the assay is as described for Method Ia, but this procedure is less sensitive than the latter, which is therefore preferable for routine use.

Method II. This procedure is a modification of the Lowry-Lopez method (Lowry and Lopez, 1946) for use in the presence of acid-labile organic phosphate. The following stock reagents are required: (A) 2% (v/v) Triton X-100 in 1M sodium acetate buffer, pH 4.0; (B) and (C) as for method I. The deproteinized test sample (1 ml) containing 0.05-1.0 µmoles of orthophosphate and 3% (w/v) perchloric acid is mixed with 1M sodium acetate (0.7 ml) and water (0.3 ml) to give a solution with a final pH of 3.8 to which the reagents A, B and C (1 ml of each) are added in that order (final volume: 5 ml; final pH: 3.8). The assay mixtures are allowed to stand at room temperature for 15 min and their extinction at 650 nm measured spectrophotometrically against a reagent blank using 1 cm pathlength cells.

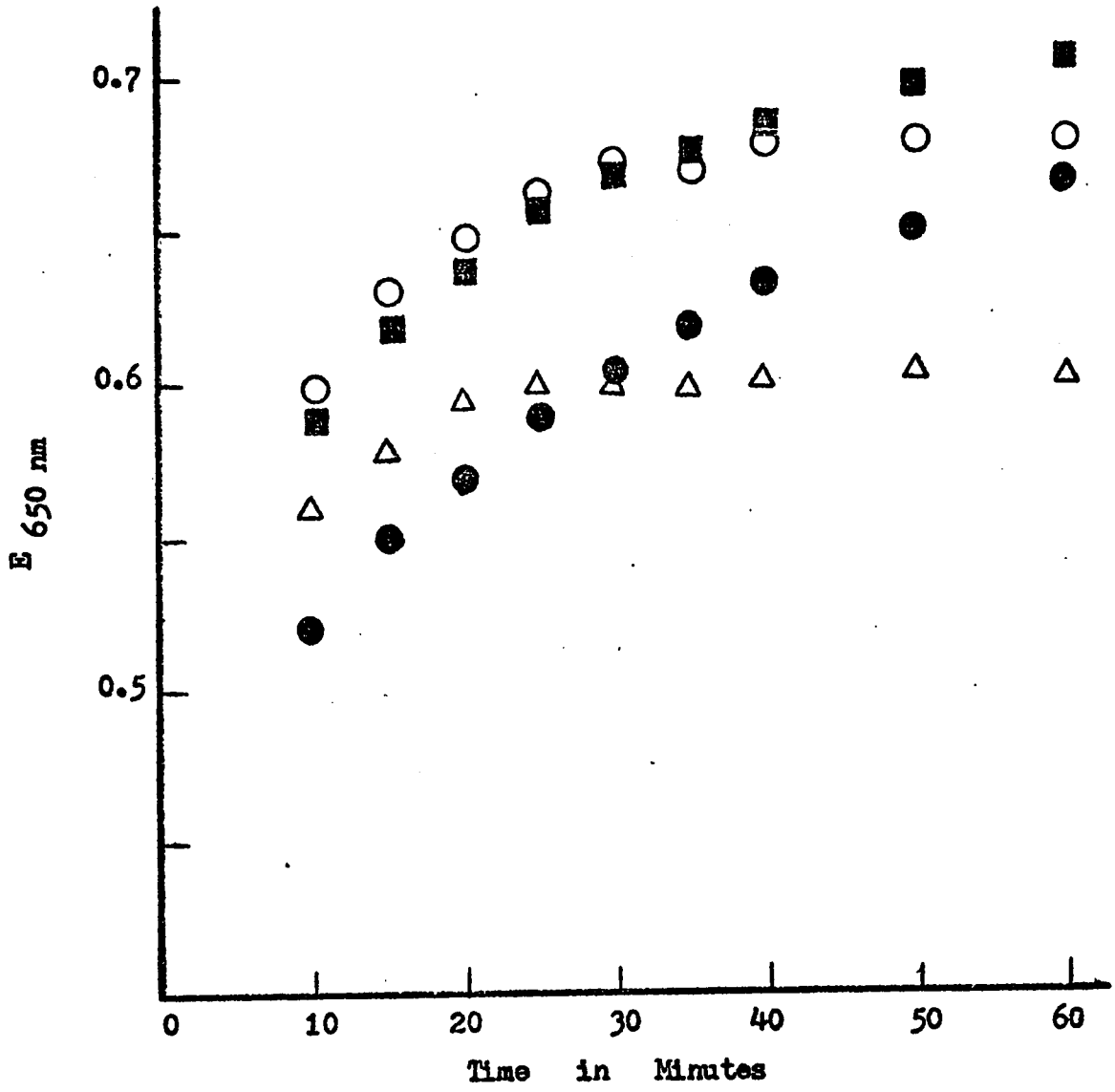


Figure 1: Effect of sulphuric acid concentration on the rate of colour development. The reaction mixture (5 ml) contained: 0.4 µmole KH_2PO_4; 0.2 ml 10% (v/v) Triton X-100; 1 ml ammonium molybdate (1%, v/v); 1 ml of reducing agent (reagent C, in methods) and 1 ml of H_2SO_4 of the indicated concentrations: (●), 0.25 N; (■), 0.1 N; (○), 0.05 N; (△), 0.025 N. The reactions were started by the addition of reducing agent and the extinction at 650 nm was recorded at the time indicated.

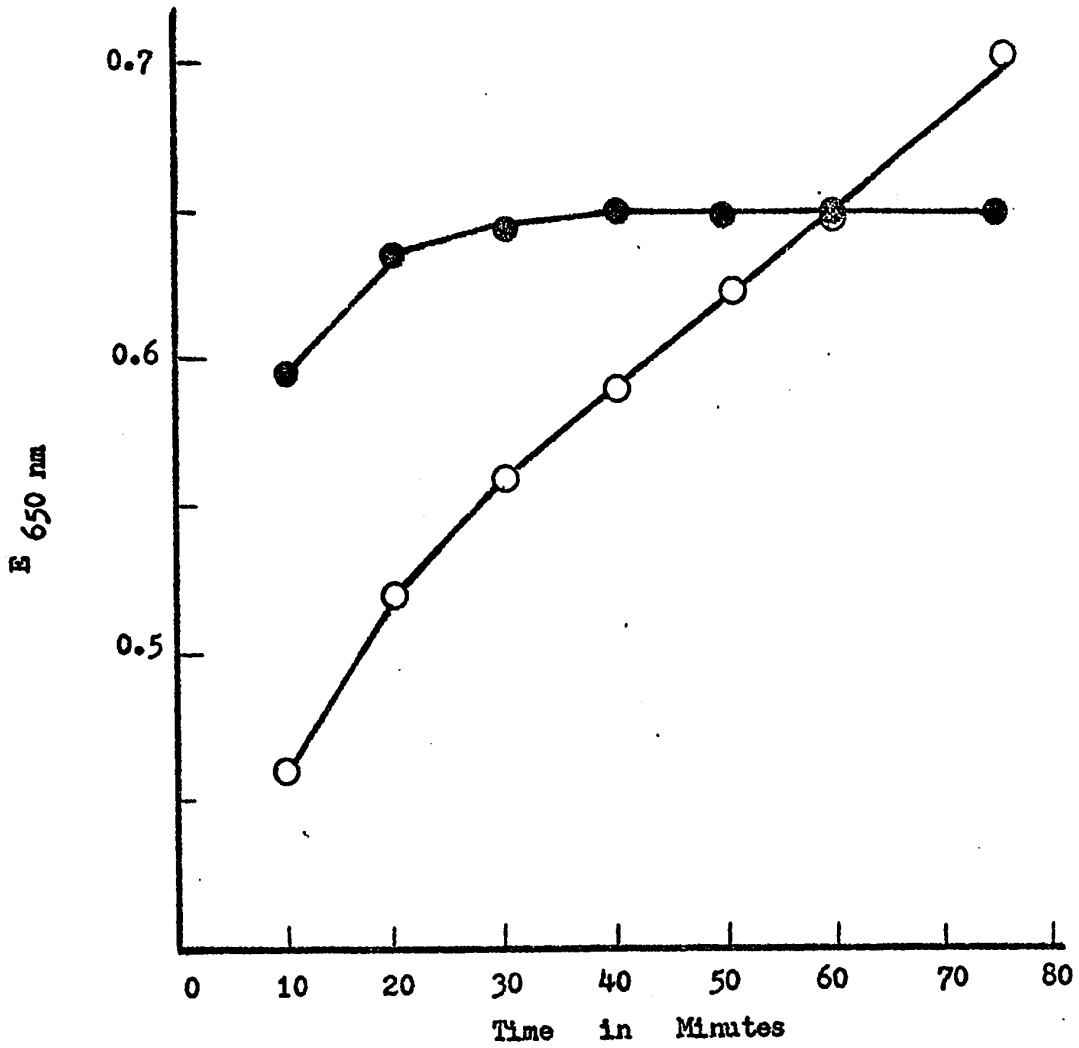


Figure 2: Effect of $HClO_4$ on the rate of colour development (Method Ia) in the absence of glycine as buffer. Inorganic phosphate (0.4 µmole) was determined by method Ia, except reagent A did not contain glycine. The extinction at 650 nm was recorded at the time indicated in the presence of $HClO_4$: (●), 0% and (○), 0.6% (w/v of total mixture).

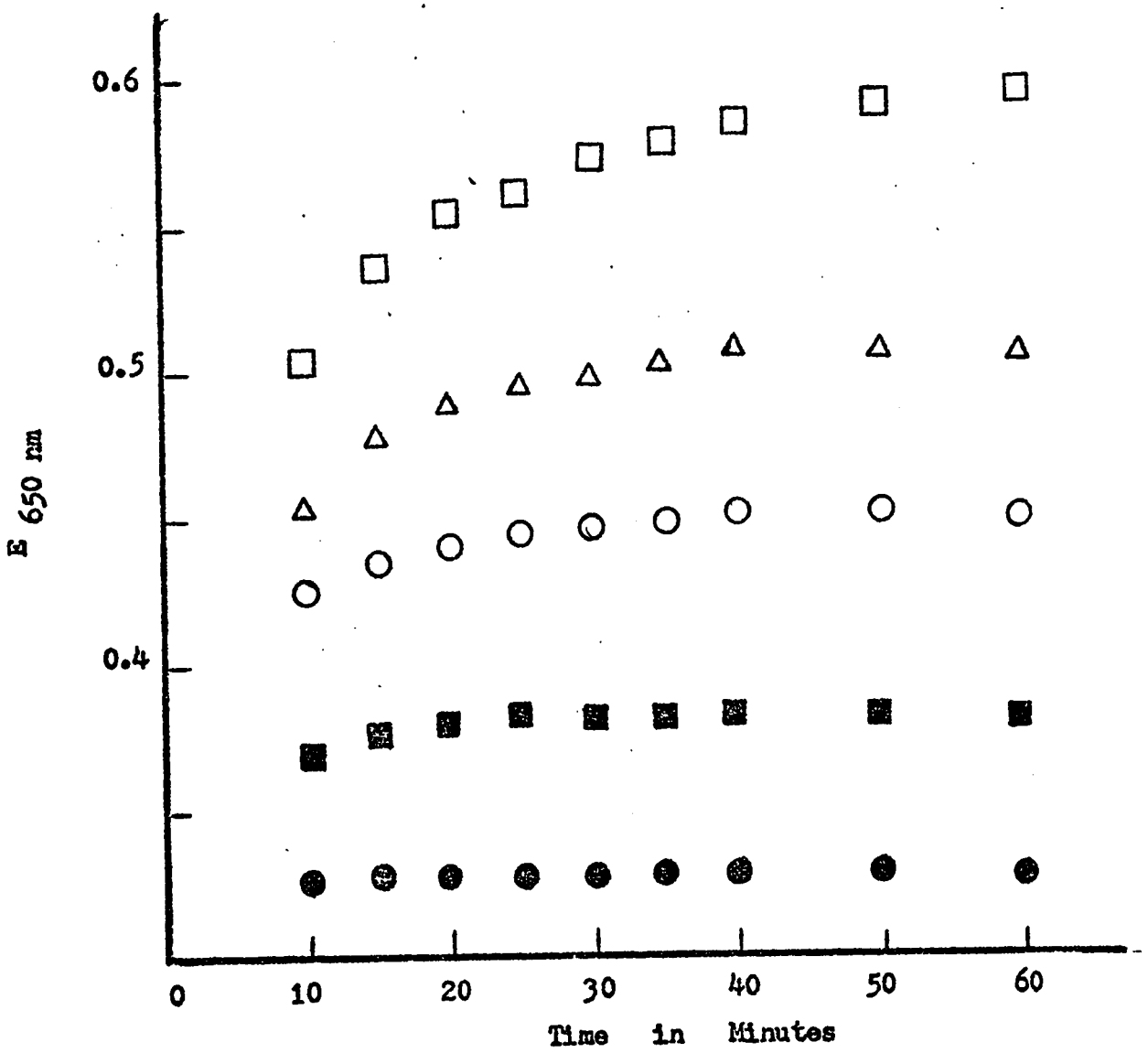


Figure 3: Effect of $HClO_4$ on the rate of colour development (Method Ia). Inorganic phosphate (0.4 µmole) was determined by method Ia (total vol. 5 ml) and the extinction at 650 nm was recorded at the time indicated, in the presence of $HClO_4$: (●), 0%; (■), 0.2%; (○), 0.4%; (△), 0.6%; (□), 0.8% (w/v of total mixture).

Results and Discussion

Method I (Modified Fiske and SubbaRow assay). The yellow precipitate formed in the Fiske and SubbaRow assay at final concentrations of Triton X-100 up to about 0.05% (v/v) redissolved when the amount of detergent was increased to 0.1% (v/v) or higher. The resulting yellow solution had an extinction maximum at 355 nm that was proportional to orthophosphate concentration in the range 2-10 nmoles/ml. Addition of reducer caused development of the molybdenum blue color, but the rate of the reaction was very sensitive to changes in pH. By keeping the ammonium molybdate concentration constant and varying the sulphuric acid concentration (Fig. 1), a concentration of the latter (0.05N) that gave the optimum rate of colour development was chosen for preparation of the acid molybdate reagent (reagent B).

Fig. 2 shows that the amount of perchloric acids normally used in the deproteinization of enzymic reactions interfered with this system. The mixture was therefore buffered with 0.02M glycine to minimize the effect of the perchloric acid used to stop the phosphatase and pyrophosphatase catalyzed reactions (see section III B) and a study was made of the effect of perchloric acid on the development of the molybdenum blue color in these conditions (Fig. 3). The amount of perchloric acid used to stop phosphatase-catalyzed reactions was then chosen to give a final concentration in the orthophosphate assay mixture of 0.6% (w/v), since this provided the best combination of sensitivity and rate of color development.

The standard curve for Method Ia (Fig. 4) shows that the

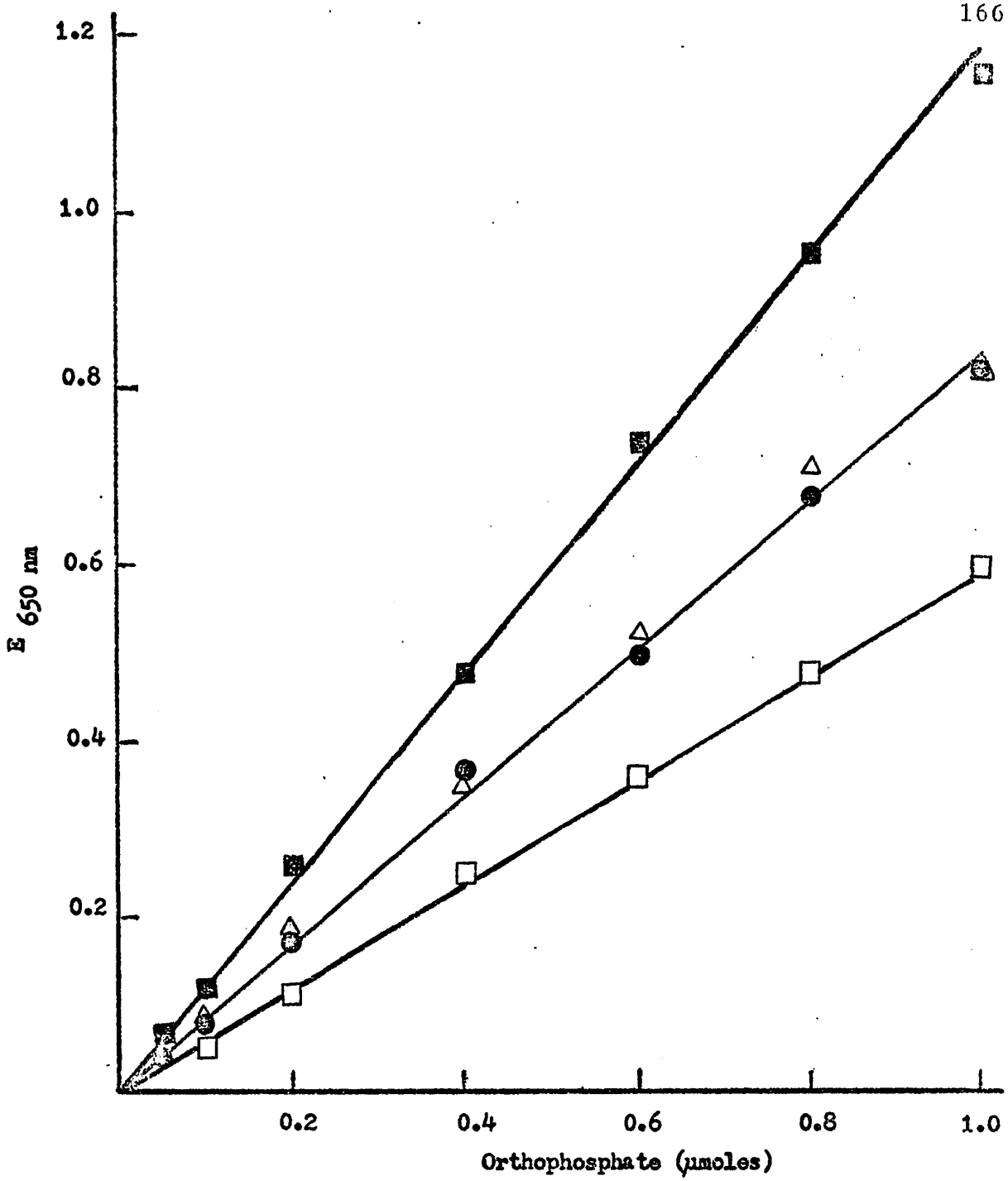


Figure 4: Standard curves for orthophosphate determination: (■), Method Ia; (□), samples from Method Ia diluted with an equal volume of distilled wather; (△), Method Ib; (●), Method II.

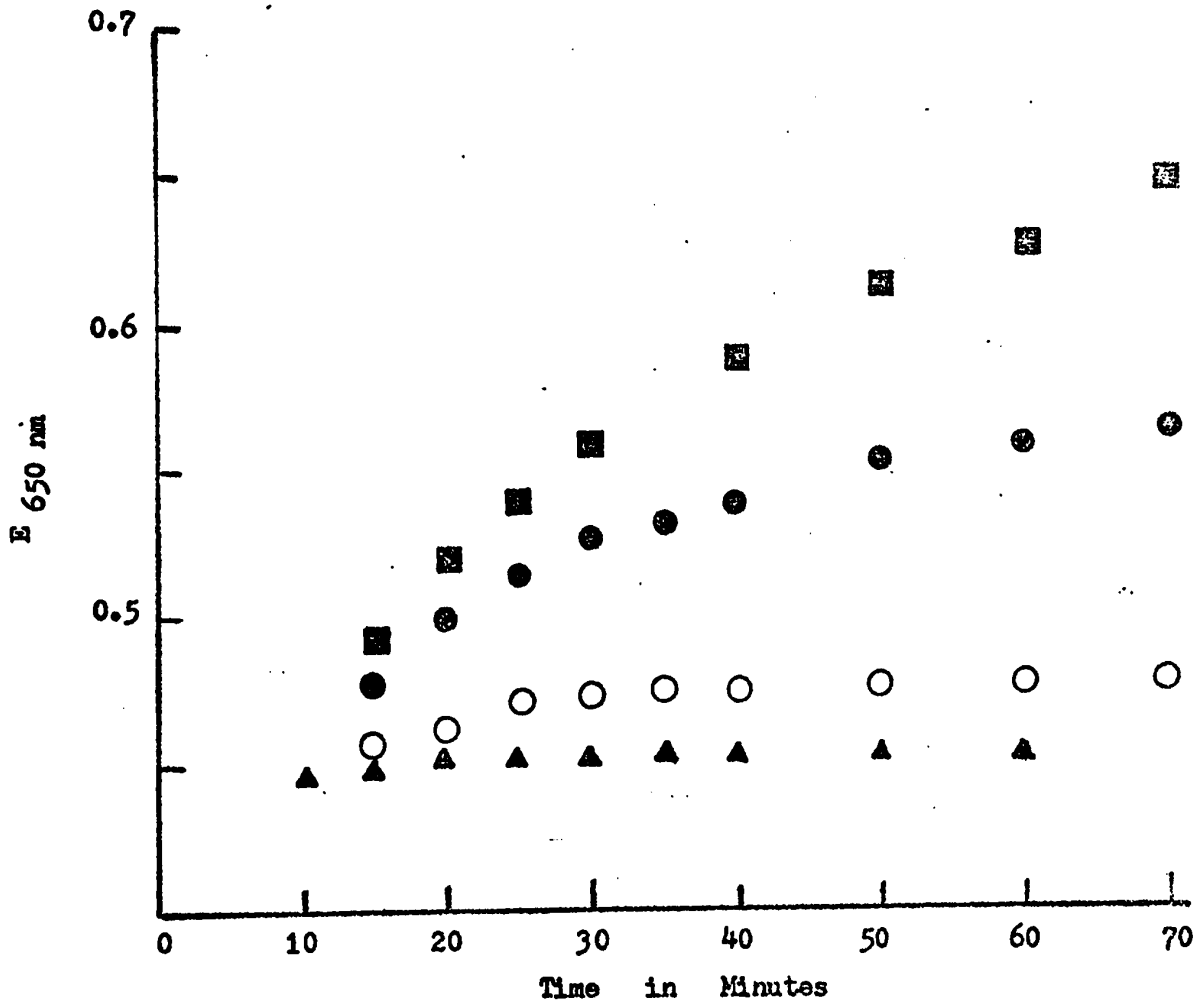


Figure 5: Effect of $HClO_4$ on the rate of colour development (Method Ib). Inorganic phosphate (0.5 µmole) was determined by Method Ib (total vol. 5 ml) and the extinction at 650 nm was recorded at the time indicated in the presence of $HClO_4$: (▲), 0.6%; (○), 0.8%; (●), 1.2%; (■), 1.6% (w/v of total mixture).

extinction at 650 nm is proportional to orthophosphate concentration in the range 0.05-1.0 μ moles in the assay.

If the samples contain sucrose as, e.g., after isolation of mitochondria in sucrose solutions, glycine is precipitated unless reducer is added to the test solution within 2 to 3 min. If large numbers of assays are to be performed, it is then more convenient to add the reducer before the acid molybdate reagent (Method 1b). This method is also less sensitive to change in perchloric acid concentration (Fig. 5) than method Ia (Fig. 3). Sucrose does not interfere with the modified procedure at concentrations up to 0.12M, but there is a loss of sensitivity compared with Method Ia (Fig. 4).

2-Mercaptoethanol has little effect on the results of the assay at final concentrations up to 6 μmoles/ml and only interferes seriously when its final concentration is above 10 μ moles/ml. Calcium, magnesium, manganese and sodium chlorides have no effect on the assay at final concentrations up to 25mM (the highest tested).

The maximum permissible concentration in the assay of Triton X-100 additional to that already present in the reagents (0.4% (v/v), final concentration) was determined in the presence of 0.5 μ moles of orthophosphate. No effect was observed up to a total concentration of additional Triton X-100 of 0.4-0.5% (v/v) in the assay mixture (equivalent to 2.0-2.5% (v/v) in the 1 ml test sample), but higher concentrations interfered with both Method Ia and Method Ib.

It should be noted that after colour development the extinc-

tion at 650 nm is still proportional to phosphate concentration when the assay solution from Method Ia is diluted with an equal volume of water (Fig. 4).

Method II (modified Lowry-Lopez assay). Further study of Method I (above) showed that the colour intensity was still proportional to phosphate concentration at pH 4.0, so a modification of the Lowry-Lopez procedure (Lowry and Lopez, 1946) was developed for use in the presence of acid-labile organic phosphate.

Method II differs from method Ia as follows: (i) 1M sodium acetate buffer, pH 4.0 replaces 1M glycine; (ii) the pH of the test solution containing the perchloric acid used to stop the enzyme reaction is adjusted to 3.6-4.0 by addition of an aliquot to a large excess of 1M sodium acetate; (iii) the extinction of the samples at 650 nm is determined after 15 min instead of 30 min.

Fig. 4 shows the standard curve for determination of orthophosphate in these conditions. The sensitivity is similar to that of Method Ib. 20mM Magnesium, sodium or calcium chloride or 0.12M sucrose did not interfere with the assay at the final concentrations indicated (the highest tested). (10mM-2-mercaptoethanol (highest tested) had no effect on the assay).

Method II is extremely insensitive to changes in Triton X-100 concentration and the extinction at 650 nm of assay mixtures containing 0.5 µmoles of orthophosphate was unaffected up to a final concentration of the detergent of 5% (v/v) in addition to that already present in the reagents.

In the standard conditions, colour development is complete within 10 min and no further change occurs during an additional 60 min. It should be noted that the final pH of the assay mixture should not exceed 4.0 or the rate of the reaction becomes very slow: the optimum pH range is 3.6-4.0.

III. B. Properties of rat liver mitochondrial inner membrane phosphatases

In section II C, it was shown that mitochondrial polynucleotide phosphorylase was exclusively localized in the inner membrane of rat liver mitochondria and that the products of the phosphorolysis of polyribonucleotides are nucleoside diphosphates. Preliminary examination of interfering enzymes in the inner membrane preparations revealed the presence of a nucleoside diphosphatase activity.

Ernster and Jones (1962) detected nucleoside diphosphatase activity in rat liver mitochondria which had a different substrate specificity from the more abundant microsomal enzyme. Two alkaline pyrophosphatases which also had nucleoside diphosphatases activity were extracted from rat liver mitochondria, by repeated freezing and thawing, and purified by Irie et al. (1970). The location of these enzymes is not known. An alkaline pyrophosphatase was localized in the inner membrane of rat liver mitochondria (Schnick and Butler, 1969). However, only inorganic pyrophosphate was used for assay and very little work was done on the properties of this membrane-bound enzyme.

In order to follow the removal of the nucleoside diphosphatase activity during the purification of the polynucleotide phosphorylase, it was necessary to study the properties of this phosphatase in some detail.

Methods

Rat liver mitochondrial inner membranes were prepared as described in section II.

Phosphatase activity was determined by following the release of inorganic phosphate from suitable substrates. The standard assay medium (1 ml) contained: 0.1M-tris-HCl buffer, pH 8.0; 5mM-$MgCl_2$; 1mM-2-mercaptoethanol; 1mM-ADP; 0.1% (v/v) Triton X-100. The reaction mixtures were equilibrated at 37° for 5 min and reactions were started by the addition of inner membranes. Incubation was at 37° for 30 min in a water bath-shaker. The reaction was stopped with 1 ml of cold 6% (w/v) $HClO_4$ and allowed to stand at 0° for 10 min. The mixtures were centrifuged (clinical centrifuge) at full speed for 2 min and 1 ml sample was taken for inorganic phosphate determination. Controls without enzyme were carried out in all cases and the assay results corrected accordingly.

Inorganic phosphates were determined by either method Ib or method II as described in section III A.

Results

Enzymic properties of rat liver mitochondrial inner membrane phosphatases

Fig. 1 shows the enzyme saturation curve of inner membrane phosphatase. In the standard assay condition the rate of hydrolysis of ADP was proportional to enzyme concentration up to 0.55 µmole of inorganic phosphate released.

The pH optimum of the enzyme was at 8.0 with either ADP or CDP as substrate (Fig. 2).

The hydrolysis of adenine nucleotides does not seem to require divalent cation for activity (Table 1 and Fig. 3) and, with ADP as substrate, the enzymic activity was inhibited by in-

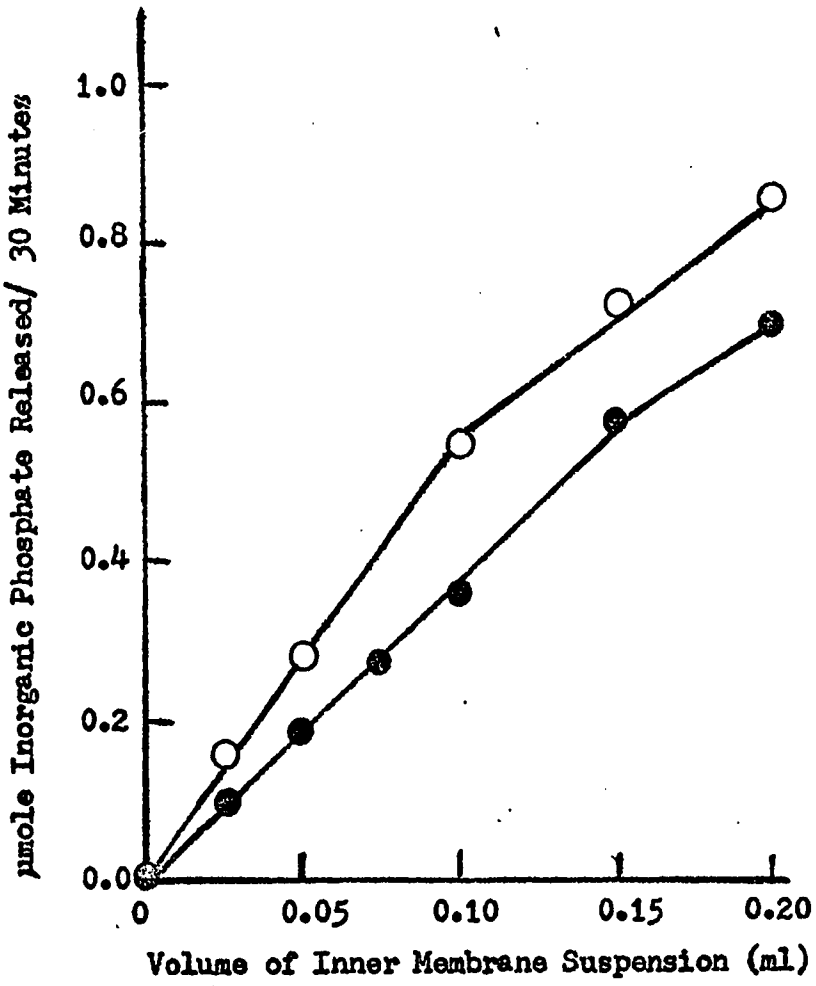


Figure 1: Effect of enzyme concentration on the hydrolysis of ADP by inner membrane phosphatases. The hydrolysis of ADP was assayed in the standard conditions with the indicated volume of inner membrane suspension. Two preparations of inner membrane were used and the phosphate released in each case was determined by different methods developed in Section III A. The phsophate released was determined by: (●), method Ib (protein, 4 mg/ml); (○), method II (protein, 4.4 mg/ml).

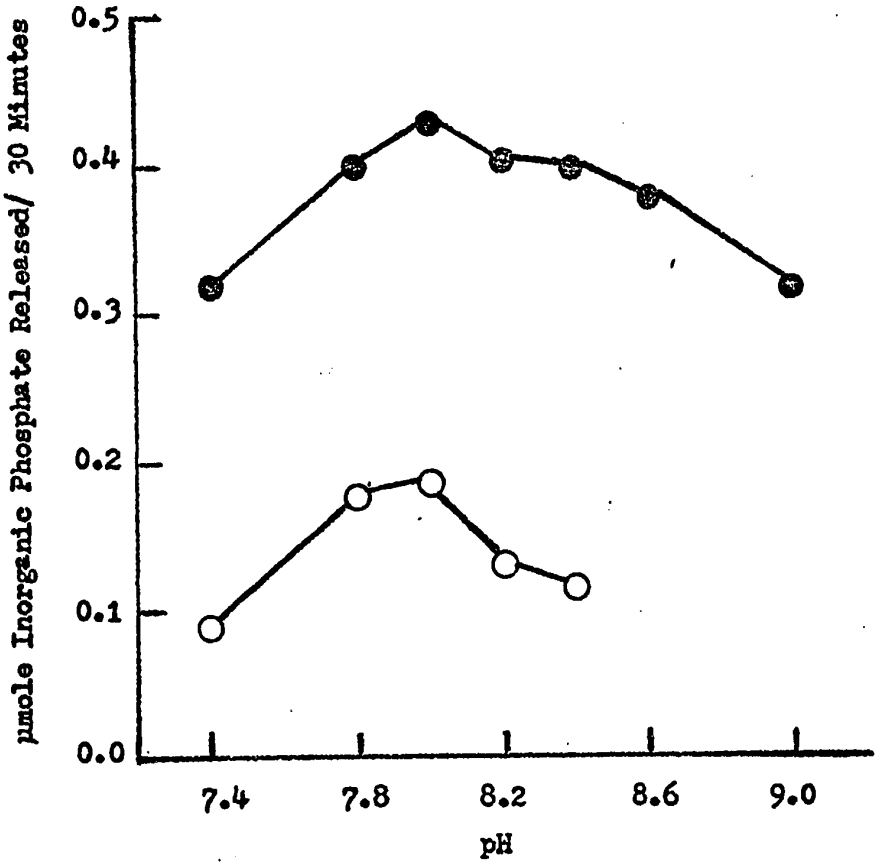


Figure 2: Effect of pH on the hydrolysis of ADP and CDP. The hydrolysis of ADP and CDP was assayed in the standard conditions with 10 mM-tris-HCl buffers of the indicated pH:(●), ADP;(○), CDP.

TABLE 1

Substrate specificity of mitochondrial inner membrane phosphatases in the presence and absence of Mg^{2+}

Phosphatase activity of rat liver mitochondrial inner membrane was measured as described in the methods section in the presence and absence of 5mM-$MgCl_2$ with the indicated substrates

Substrates	µmole orthophosphate released/30 min incubation			
	*Exp. I		*Exp. II	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
ATP	0.94	0.84	1.07	1.08
UTP	-	0.44	0.02	0.44
ADP	0.45	0.33	0.49	0.40
CDP	0.05	0.13	0.02	0.09
GDP	0.08	0.31	0.01	0.18
UDP	0.05	0.31	0.03	0.20
AMP	0.11	0.19	0.23	0.43
UMP	-	-	0.30	0.53
PP_i	0.06	1.78	0.01	1.53
p-nitrophenylphosphate	-	-	0.03	0.02

* Separate membrane preparations were used for Exp. I and Exp. II.

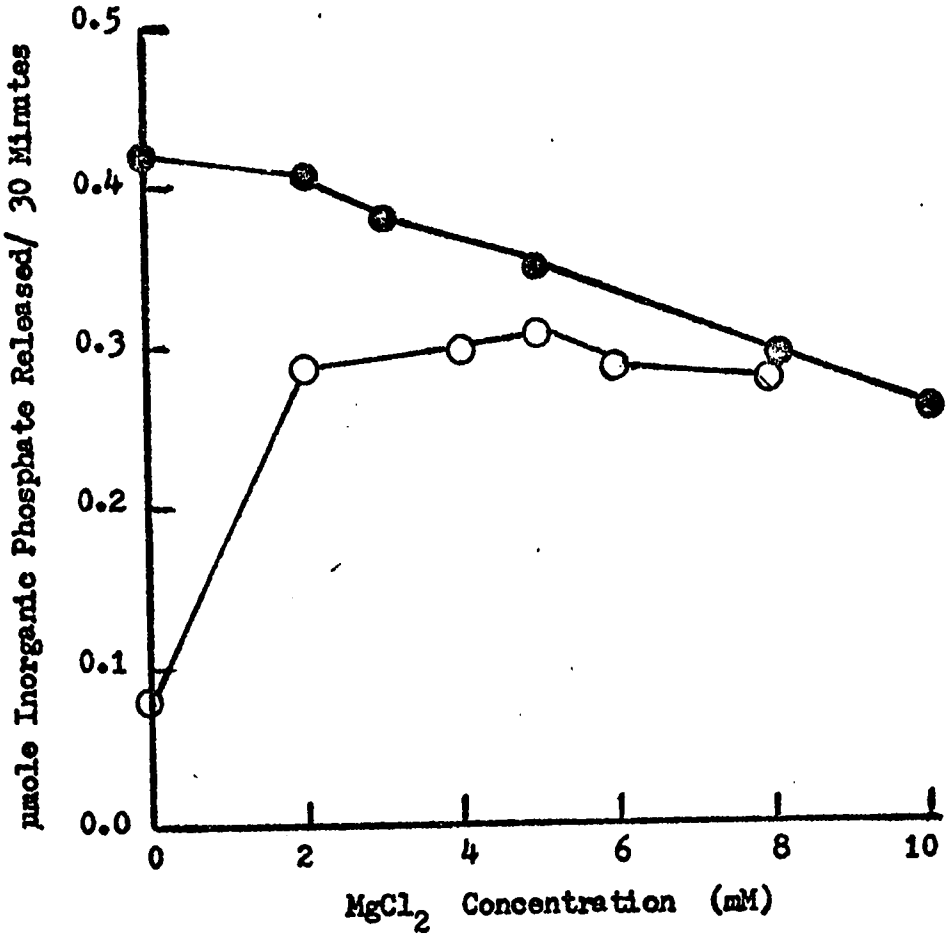


Figure 3: Effect of $MgCl_2$ on the hydrolysis of ADP and GDP. The hydrolysis of ADP and GDP was assayed in the standard conditions with the indicated $MgCl_2$ concentrations: (●), ADP; (○), GDP.

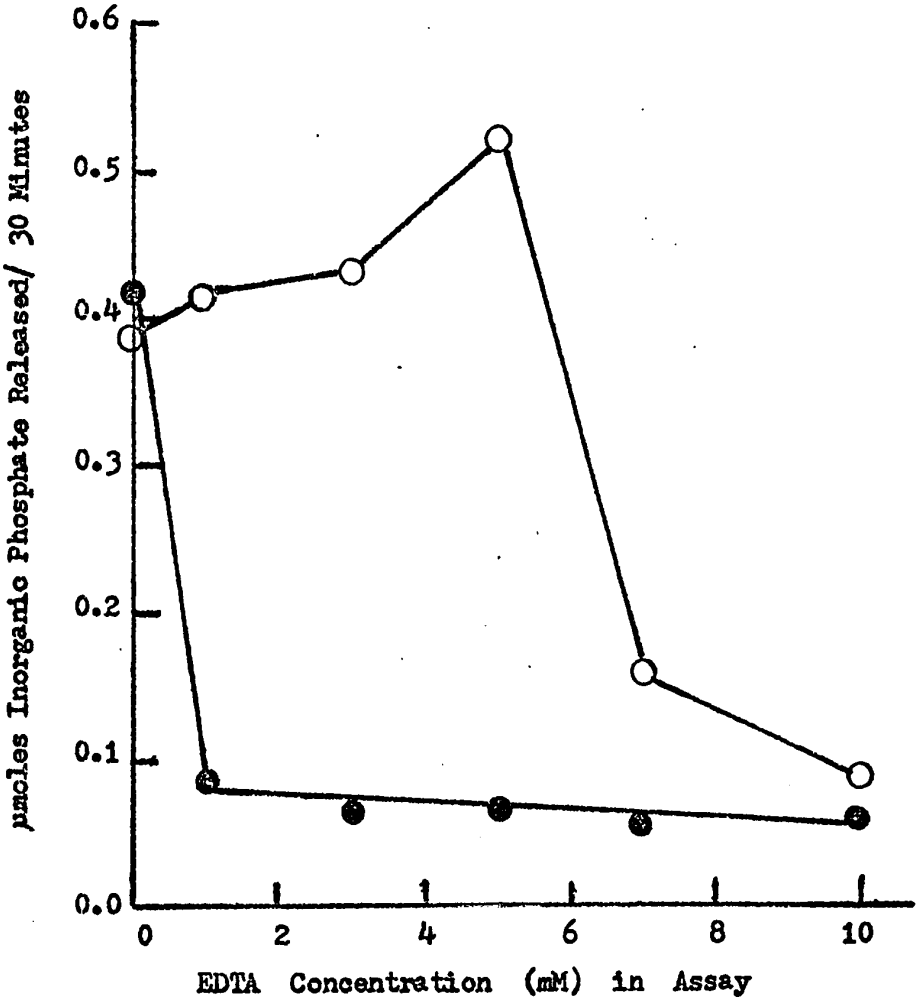


Figure 4: Effect of EDTA on the hydrolysis of ADP by inner membrane phosphatase in the presence and absence of Mg^{2+}. The hydrolysis of ADP was assayed in the standard conditions with the indicated concentrations of EDTA, in the absence of Mg^{2+} (•) and in the presence of 5 mM Mg^{2+} (○).

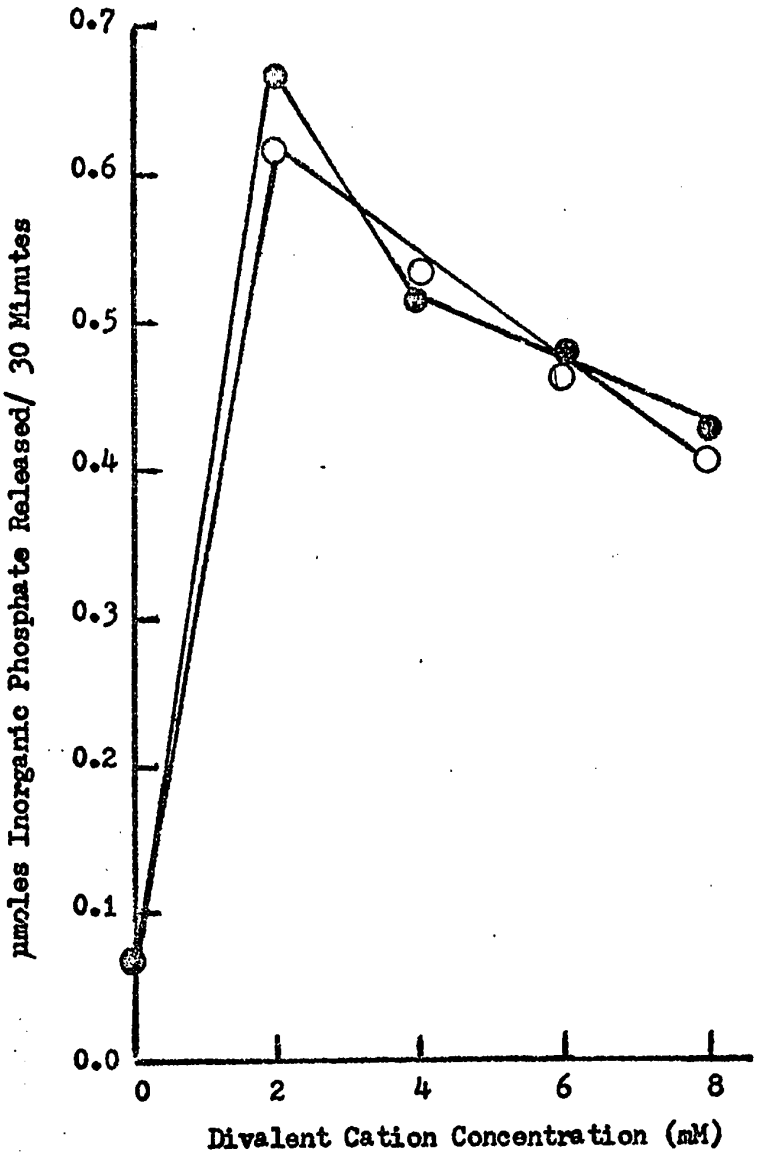


Figure 5: Effect of $MgCl_2$ and $CaCl_2$ on the hydrolysis of ADP by inner membrane phosphatase in the presence of EDTA. The hydrolysis of ADP was assayed in the standard conditions in the presence of 1 mM EDTA and the indicated divalent cation concentrations: (●), Mg^{2+}; (○), Ca^{2+}.

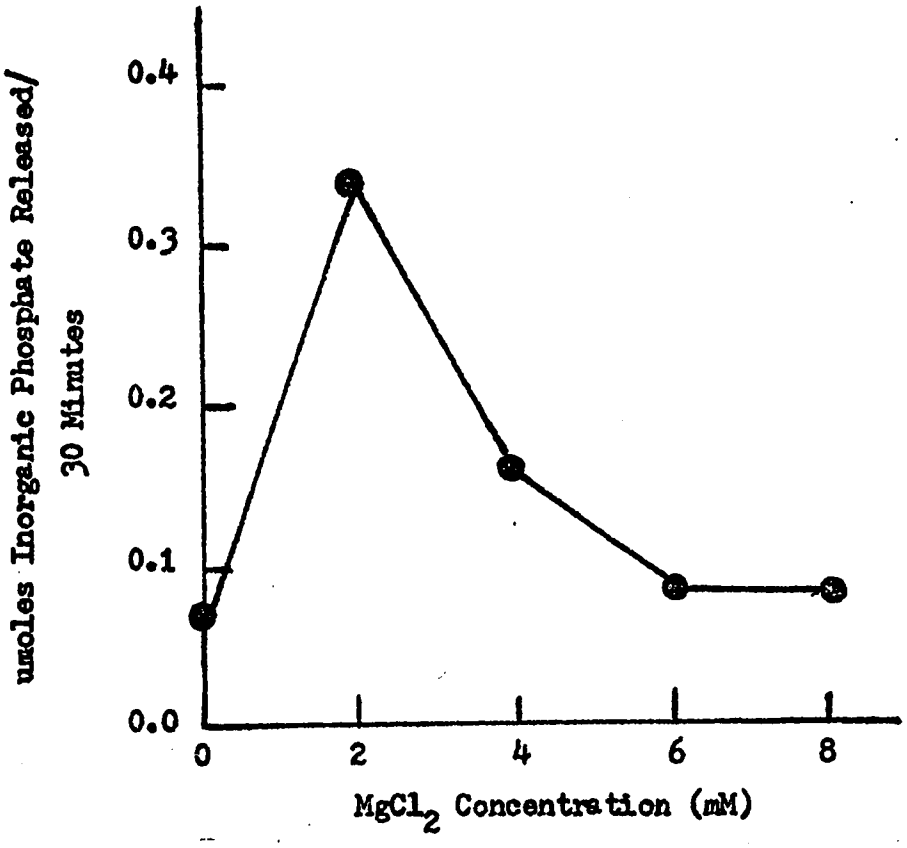


Figure 6: Effect of $MgCl_2$ on EDTA treated inner membrane phosphatase activity. EDTA was added to 1 ml of inner membrane suspension (4 mg protein/ml) to a final concentration of 2 mM and dialyzed overnight against 2-mercaptoehanol-10mM-tris-HCl buffer, pH 7.4 (2 l). The hydrolysis of ADP was assayed in the standard conditions in the presence of the $MgCl_2$ concentrations indicated.

creasing Mg^{2+} concentrations (Fig. 3). In contrast, the hydrolysis of the other nucleotides and inorganic pyrophosphate required Mg^{2+} for activity (Table 1 and Fig. 3). This indicates that there may be at least two phosphatases present in the inner membrane preparations, one which is specific for adenine nucleotides and does not require added divalent cation for activity and, a non-specific phosphatase which required added divalent cation (Mg^{2+}) for activity. The enzymes did not hydrolyse p-nitrophenylphosphate (Table 1) and, therefore, are alkaline pyrophosphatases and not non-specific alkaline phosphatases.

The properties of the "divalent-cation-independent" phosphatase activity were examined further. The hydrolysis of ADP in the absence of divalent cation was completely inhibited by the presence of EDTA (Fig. 4). However, when the enzyme was assayed in the presence of 5mM $MgCl_2$, the addition of EDTA up to 5mM stimulated enzymic activity and, higher EDTA concentration produced inhibition (Fig. 4). The stimulation by EDTA below 5mM is due to removal of Mg^{2+} which inhibits hydrolysis of ADP (Fig. 3). In the presence of 1mM-EDTA in the assay, phosphatase activity could be restored to the original level by either Mg^{2+} or Ca^{2+} (Fig. 5). These results suggested that the "divalent-cation-independent" phosphatase that is specific for adenine nucleotides contains bound divalent cations. The data given in Fig. 6 confirmed this suggestion. The EDTA-treated membrane required divalent cation for activity.

The inner membrane phosphatases also hydrolyse AMP and UMP (Table 1) and this may be due to another enzyme, probably 5'-

nucleotidase which has been shown to be located mainly in the plasma membrane of the cell (Song and Bodansky, 1967). The substrate specificity, for nucleoside diphosphates, of the mitochondrial inner membrane phosphatases was very similar to that shown for whole mitochondria (Ernster and Jones, 1962). The ease of degradation of nucleoside diphosphates by mitochondrial phosphatases, in the presence of Mg^{2+}, was ADP > UDP ≡ GDP > CDP (Table 1) and, was UDP > GDP >> ADP > CDP for the microsomal enzume (Ernster and Jones, 1962). The ADPase activity was different from that reported for plasma membrane (Wattiaux-De-Coninck and Wattiaux, 1969). The plasma membrane ADPase had a lower pH optimum and required divalent cations for activity.

Two alkaline pyrophosphatases have been isolated and purified from rat liver mitochondria (Irie et al., 1970). The enzymes were solubilized by repeated freezing and thawing of whole mitochondria. These pyrophosphatases also hydrolysed nucleotides. However, most of the properties of these purified enzymes were obtained with inorganic pyrophosphate as substrate. I have confirmed the authors' finding that repeated freezing and thawing only removes part of the alkaline pyrophosphatase activity from whole mitochondria. For these reasons, it is difficult to compare the inner membrane (pyro)phosphatases with the purified enzymes. They may be different enzymes. However, the pH stabilities at 25° and the pH optimum of the inner membrane enzymes reported here are similar to those found by Irie et al. (1970).

IV. Discussion

The data presented here show that polynucleotide phosphorylase occurs in animal cells. The enzyme is definitely present in animal mitochondria and possibly the nuclei. However, the nuclear enzyme may be due to mitochondrial contamination. The mitochondrial enzyme of rat liver is located exclusively in the inner membrane of the organelles. The nuclear enzyme, as isolated, is also associated with membrane fragments whose origin is uncertain. The rat liver mitochondrial polynucleotide phosphorylase has been solubilized and partially purified. The partially purified enzyme is particulate and contained little or no lipids.

Both the partially purified mitochondrial and nuclear (membrane associated) polynucleotide phosphorylases catalysed phosphorolysis of polyribonucleotides and are associated with ADP/P_i and ATP/P_i exchange activities. They do not catalyse polymerization of nucleoside diphosphates. The rat liver mitochondrial inner membranes contain phosphatases that degrade nucleotides and inorganic pyrophosphate but not p-nitrophenylphosphate. The phosphatases were completely removed during purification. The purified mitochondrial enzyme is also contaminated by an endonuclease and adenylate kinase, but it was confirmed that the failure to detect polymer formation was not due to nuclease activity.

The rat liver mitochondrial inner membrane polynucleotide phosphorylase was solubilized with Triton X-100 and purified by adsorption on Mn-RNA gel and DEAE-cellulose column chromatography. The enzyme was eluted from the DEAE-cellulose column with 0.25M-NaCl.

The eluate was clear and is slightly cloudy after ammonium sulphate concentration, but large particles containing all the polynucleotide phosphorylase activity were formed during dialysis against low ionic strength buffer (see p. 76). A similar tendency to aggregate as a result of changes in the ionic strength of the medium had been observed with beef heart mitochondrial cytochrome oxidase (Sun et al., 1968), erythrocyte membrane proteins (Mazia and Ruby, 1968; Blumenfeld et al., 1970), hydrophobic lipoproteins from Neurospora crassa and Hydrogenomonas facilis (Kuehn et al., 1969; Shannon and Hill, 1971) and guinea-pig liver polynucleotide phosphorylase (See and Fitt, 1970; cf. p.117).

The tendency of the mitochondrial polynucleotide phosphorylase to aggregate suggested that its overall amino acid composition is similar to the erythrocyte membrane protein and other membrane structural proteins (Mazia and Ruby, 1968) which contain large hydrophobic residues. The erythrocyte membrane protein had an overall amino acid composition similar to that of other membrane structural proteins and contained about 30% non-polar amino acid (Mazia and Ruby, 1968). It has been calculated, from the properties of known proteins, that the tendency of proteins to aggregate depends on the relative concentration of polar and non-polar amino acid residues (Fischer, 1964) and aggregation is favoured when the hydrophobic residues exceed 30% (Van Holde, 1966). The presence of large numbers of non-polar amino acid residues in the partially purified polynucleotide phosphorylase will have to be confirmed by amino acid analysis but it would explain the observation that solubilized preparations of the enzyme are very unstable.

The enzyme is relatively stable in the final particulate state. In the aggregated state, most of the hydrophobic residues of the proteins are buried and out of contact with the aqueous medium (Fischer, 1964; Van Holde, 1966) and, thus, stabilize the protein (enzyme) molecules. The hydrophobic residues that are exposed during solubilization and purification are most probably associated with lipids in the membrane bound state. The requirement of the presence of salts for aggregation can be explained by neutralization of charge groups. The degree of aggregation is dependent on the ionic strength of the medium; the higher the salt concentration, the greater the aggregation (Mazia and Ruby, 1968). The salt-promoted aggregation is not reversible (personal observation; Mazia and Ruby, 1968; Maddy and Kelly, 1971) even by treatment with 6M guanidine-HCl (Maddy and Kelly, 1971). The size of the partially purified rat liver mitochondrial polynucleotide phosphorylase formed during dialysis to remove NaCl is dependent on the protein concentration. If the individual fractions (3 ml, each) after the DEAE-cellulose column step are dialysed, instead of being concentrated with ammonium sulphate, and dissolved in the small volume of buffer recommended (Method viii), only part of the enzyme can be recovered in large particles. The rest of the polynucleotide phosphorylase can be sedimented at 100,000 g for 1 h.

The animal polynucleotide phosphorylases studied are inhibited by sodium chloride (Figs. 12 and 22). The guinea-pig nuclear enzyme, with which the effect of potassium chloride was also studied, is inhibited to the same extent by potassium chloride, so the inhibition is probably non-specifically related to the ionic

strength of the medium. The inhibition by salts may be explained by: (i) an increase in the secondary structure of the poly A, since phosphorolysis catalysed by the E. coli polynucleotide phosphorylase has been shown to be sensitive to the amount of secondary structure in the polyribonucleotides (Grunberg-Manago, 1959; Michelson, 1963); (ii) an inhibition of the enzyme itself probably due to alteration in the conformation of the protein with increasing ionic strength.

The effect of salt concentrations on the structure of poly A at neutral pHs in the range used (0 to 0.15-M-NaCl or KCl), that inhibited animal polynucleotide phosphorylase, is not known. It has been shown that there is some increase in the secondary structures of poly A and poly U, at neutral pHs, when the concentrations of alkali metal salts (LiCl, NaCl, KCl and CsCl) is increased from 0.15M to 1M (Leng and Michelson, 1968; Thrierr and Leng, 1969). The effect of salts on the secondary structures of polyribonucleotides in the presence of magnesium ions, phosphates and Triton X-100 used in the assay is once again unknown. However, it seems unlikely that the large inhibition of animal polynucleotide phosphorylase by relatively low concentration of salts (about 90% inhibition by 0.1M-NaCl) could be explained by an increase in the secondary structure of poly A in these salt concentrations, because the available evidence suggests that any such increase would be slight in these conditions. Thus, a direct effect on the enzyme seems a more probable explanation for the observed effects. The phosphorolysis of poly A catalysed by bacterial polynucleotide phosphorylases [E. coli (Grunberg-Manago,

1959), M. lysodeikticus (Hendley and Beers (1961) and Cl. perfringens (Fitt et al., 1968)] is stimulated by salt concentrations that inhibited the animal enzymes. Since the state of aggregation of the animal polynucleotide phosphorylases (both the partially purified mitochondrial and the membrane-bound nuclear enzyme) is affected by ionic strength the inhibition by salt may be caused by a decrease in accessibility of the active site of the enzyme with increase in ionic strength. Alternatively, the animal polynucleotide phosphorylase may be inhibited by chloride ions since only sodium or potassium chloride were used for studies (Fig. 12 and 22). The guinea-pig nuclear enzyme is inhibited to the same extent by sodium or potassium chloride.

The inhibition of animal polynucleotide phosphorylase by sodium (or potassium) chloride raised the question whether the enzyme is functional in vivo. I will consider this question in regard to the mitochondrial enzyme. It has been documented (Lehninger, 1965; Lehninger et al., 1967), that K^+ and Mg^{2+} constitute the bulk of the cations in the mitochondria and that most of the Mg^{2+} and Ca^{2+} are firmly bound to the mitochondrial structures. Acid soluble phosphate compounds are the main anions in the mitochondria and few chloride ions are present (Lehninger, 1967). For this reason, the possibility of inhibition by chloride ions will not be discussed. The concentration of the various ions per mitochondrion is not known. K^+ (130 nmole/mg protein) is the most abundant cation in the mitochondrion and, if free in solution, it, together with the phosphate counterions (including, free inorganic phosphate and nucleotides) might establish an ionic strength that

could inhibit the polynucleotide phosphorylase. However, it had been suggested that the bulk of the intracellular Na^+ and K^+ are bound to macromolecules and are not free in solution (Ling, 1969; Cope, 1970). Ling and Cope (1969) and Cope (1970) had shown from NMR data that 65% of the intracellular Na^+ in muscle, brain and kidney are bound. These findings had been confirmed by Czeisler et al. (1970). If this is true, polynucleotide phosphorylase may still be active in vivo. However, it must be emphasized that the mitochondrial polynucleotide phosphorylase is tightly bound to the mitochondrial inner membrane so the isolated enzyme may not necessarily reflect its true properties in vivo. A number of enzymes have been described in the literature which are inhibited by the concentration of salts thought to exist in cells. A ribonuclease isolated and purified from rat reticulocytes (Goto and Mizuno, 1971) is inhibited by increasing sodium chloride concentrations in the same manner as for the animal polynucleotide phosphorylase described in this thesis. It is inhibited 90% by 0.1M-NaCl. The crude DNA-polymerases from new born rat liver and brain are inhibited about 70-80% by 0.1M-NaCl (or KCl) (Bharucha and Murthy, 1971); and, the fatty acid synthetase system of Halobacterium cutirubrum is strongly inhibited by 1-4M-NaCl (or KCl) (Pugh et al., 1971). This extreme halophile normally grows in a medium of 25% (4.3M) NaCl (Larsen, 1967; Kushner, 1968) and a closely related bacterium H. salinarium is known to have internal K^+ and Na^+ concentrations of 4.57 and 1.37 molal, respectively (Larsen, 1967).

The partially purified rat liver mitochondrial and the membrane bound rat liver and guinea-pig liver nuclear polynucleotide

phosphorylases catalysed phosphorolysis of poly A, poly U, poly C and natural RNA but not poly G or DNA. This property is similar to that of bacterial polynucleotide phosphorylase (Grunberg-Manago, 1963). However, the animal polynucleotide phosphorylases do not catalyse nucleoside diphosphate polymerization. The absence of the polymerization reaction had been shown, at least for the partially purified mitochondrial enzyme, not to be due to the presence of interfering enzymes (section II C). The lack of the polymerization reaction in the animal enzymes cannot be explained by the physical (particulate) state of the enzymes. Bacterial polynucleotide phosphorylase immobilized on nitrocellulose membranes (Millipores) (Thang et al., 1968) and cellulose (Hoffman et al., 1970) can still catalyse nucleoside diphosphate polymerization reactions.

The animal polynucleotide phosphorylases are associated with ADP/P_i and ATP/P_i exchange activities. However, the properties of the ADP/P_i exchange activity of the rat liver mitochondrial enzyme (section II C) and the guinea-pig liver nuclear enzyme (section II D) suggested that this exchange is due to a different enzyme. This conclusion is in accordance with the hypothesis that the ADP/P_i exchange activity is an expression of the reversible polymerization and phosphorolysis reactions taking place at near equilibrium (Bon et al., 1970; Chou and Singer, 1971a; Simuth et al., 1971). The lack of nucleoside diphosphate polymerization and ADP/P_i exchange activity in animal polynucleotide phosphorylase resembles the enzyme from E. coli mutants, E. coli Q13 (Thang et al., 1967, 1969) and E. coli PR7 (Reiner, 1969a,b). The ATP/P_i exchange activity

was not studied in great detail. The ATP/P_i exchange activity associated with the guinea pig liver nuclear enzyme has properties similar to those of the ADP/P_i exchange activity apart from the difference in pH optimum and stability during storage. The ATP/P_i exchange activity associated with the partially purified mitochondrial polynucleotide phosphorylase has not been studied. It may be due to an enzyme similar to the one extracted from acetone treated rat liver mitochondria and purified by Plaut (1957). This enzyme catalysed ATP/P_i exchange as well as ADP/ATP exchange; the two exchange activities are specific for adenine nucleotides (Plaut, 1957; Chiga and Plaut, 1959). The enzyme does not catalyse ADP/P_i exchange.

The polynucleotide phosphorylase extracted and partially purified from rat liver nuclei prepared by the 2.2M-sucrose procedure had the same properties as the rat liver mitochondrial inner membrane enzyme (Table 25). The nuclear enzyme is bound to membrane particles and contained cardiolipin (section II D) which is considered to be located exclusively in the mitochondria of rat liver (Getz, 1970). Further, when nuclei were isolated by the 0.25M-sucrose procedure and purified by resuspension in 2.2M-sucrose (Table 24), the amount of polynucleotide phosphorylase per milligram DNA drops considerably. This drop in extractable polynucleotide phosphorylase activity follows the drop in cytochrome oxidase activity (a marker enzyme for mitochondrial contamination). When the nuclei were isolated by the 2.2M-sucrose procedure and purified by resuspension in 2.2M-sucrose, the nuclei obtained contained no polynucleotide phosphorylase activity, but the yield of nuclei in

this case is poor. The inability to extract polynucleotide phosphorylase from purer nuclei was confirmed with nuclei obtained by the large scale modification of Blobel and Potter procedure. This procedure produces purer and better yield of nuclei. It is of interest to note that nuclear membrane obtained from nuclei isolated by a similar procedure does not contain cardiolipin (Franke et al., 1970; Kleinig, 1970) whereas nuclear membrane obtained from nuclei isolated by a modified 2.2M-sucrose procedure (similar to method xvii) contained small amount of cardiolipin (Berezney et al., 1970;Keenan et al., 1970).

Guinea-pig nuclei prepared by the large scale modification of Blobel and Potter procedure also contain no extractable polynucleotide phosphorylase. The guinea-pig mitochondrial polynucleotide phosphorylase had the same pH optimum in tris-HCl buffer, a plateau between 8.7 to 9.2 (Fig. 25), as the guinea-pig nuclear enzyme extracted and purified from nuclei prepared by the 2.2M-sucrose procedure.

The amount of mitochondria recovered in the nuclear pellet prepared by the 2.2M-sucrose procedure was 1-3% of the total liver mitochondria (Tables 22 and 23).

The data presented above suggested that the polynucleotide phosphorylase extracted from nuclei prepared by the 2.2M-sucrose procedure is from contaminating mitochondria. However, the amount of mitochondria recovered in the nuclear pellet (1-3% of the total liver mitochondria) is insufficient to account for the amount of polynucleotide phosphorylase extracted from these nuclei (Tables 25 and 26) (see p. 146-8 for full discussion). Thus, the location of the polynucleotide phosphorylase extracted from the 2.2M-sucrose

nuclei is still uncertain. The "nuclear" enzyme could not be due to blood contamination nor from other intracellular organelles. Nuclei isolated from perfused liver (by the 2.2M-sucrose procedure) produced similar amount of extractable polynucleotide phosphorylase as non-perfused liver (section II D). There were no detectable polynucleotide phosphorylase activities in other cell organelles (glycogen particles, microsomal fraction, plasma membrane and the soluble fraction) apart from mitochondria (Table 25). Polynucleotide phosphorylase activity has not been detected in peroxisomes and lysosomes (section II C).

The data presented in this thesis show that polynucleotide phosphorylase is definitely present in the mitochondria and may be present in the nuclei. There were no detectable polynucleotide phosphorylase activity in the other cell organelles studied but the absence of the enzyme has to be confirmed by more rigorous search with different experimental conditions.

The localization of animal polynucleotide phosphorylase in the mitochondrial inner membrane and its widespread distribution in animal species and rat tissues raise again the problem of the uncertain biological role of the enzyme. The physiological function that had been proposed for bacterial polynucleotide phosphorylase had been discussed briefly in the introduction (p. 31). The most favoured role for the enzyme is the degradation of m-RNA. This was based on the observation that polynucleotide phosphorylase degraded single stranded RNAs much faster than RNAs with greater secondary structures and that the degradation of pulse labelled RNA in the presence of inorganic phosphate produced small amount of nucleoside diphosphates (see p.31).

The main problem with this hypothesis is that polynucleotide phosphorylase degrade RNA from the 3' to 5' direction, whereas, m-RNA had been shown to be inactivated from the 5' to 3' direction (Morikawa and Imomato, 1969; Morse et al., 1969a,b). This problem could be overcome if there was an endonuclease present in bacteria which could degrade m-RNA to produce oligonucleotides with free 3'-hydroxyl terminal. Bacterial polynucleotide phosphorylase will not phosphorolyse oligonucleotides with 3'-phosphate terminal which are inhibitors of the enzyme (see p. 15). No such endonuclease had been found in bacteria, so far (Morikawa and Imomato, 1969; Morse et al., 1969a,b; Duffy et al., 1972). Rat liver mitochondria contain such an endonuclease but, unfortunately, most of the enzymes are localized outside the inner membrane-matrix fraction (Morais, 1969) where all the polynucleotide phosphorylase are located. However, evidence has been presented that suggests the in vivo degradation of unstable RNA in B. subtilis may be mainly due to phosphorolytic cleavage (Duffy et al., 1970). The degradation of unstable RNA in E. coli may be mainly due to hydrolytic cleavage (Chaney and Boyer, 1972). Since both these bacteria contain polynucleotide phosphorylase, the data presented by Boyer and his co-workers (Chaney and Boyer, 1972; Duffy et al., 1972), suggested that the role of polynucleotide phosphorylase may be more subtle than so far realized.

The rat liver mitochondrial polynucleotide phosphorylase is exclusively localized in the inner membrane (Tables 3 and 4). Most of the bacterial polynucleotide phosphorylases have been isolated in the soluble fraction of the bacterial extracts (Grunberg-

Manago, 1963). However, drastic procedures, involving the use of a French press (Kimhi and Littauer, 1967), grinding with alumina (Fitt and Wille, 1969) or prolonged sonication (Gajda et al., 1970) were used to prepare the bacterial enzymes. The bulk of the polynucleotide phosphorylase in S. faecalis was bound to the plasma membrane when more gentle methods were used (Abrams and McNamara, 1962). In this case, protoplasts were prepared and then broken by gentle osmotic lysis. The membrane bound enzymes of S. faecalis and M. lysodeikticus were easily removed by repeated washings with buffers (Zaror de Behrens and Fitt, unpublished results; Munoz et al., 1968; Ellar et al., 1971). The polynucleotide phosphorylase of H. cutirubrum had also been shown to be membrane bound and could be released by sonication (Peterkin and Fitt, 1971). Polynucleotide phosphorylase had also been found to be partly localized in the ribosomes of E. coli (Wade and Lovett, 1961) and Ps. aeruginosa (Strasdine et al., 1962). The ribosome-bound enzyme was also easily removed by various mechanical treatments (Strasdine et al., 1962).

In the case of the animal polynucleotide phosphorylases, their presence in the inner mitochondrial membrane, the site of the enzymes and electron carriers of the respiratory chain, suggests a further possible function: control of the level of ADP in the neighbourhood of the phosphorylation sites via a cycle involving (i) ADP; (ii) an ATP polymerase similar to those found in animal cells (Edmonds & Abrams, 1960, 1962; Burdon, 1963; Ventkataraman & Mabler, 1963; Klemperer, 1963, 1965; Hyatt, 1967a,b; Gurgo et al., 1970; Dravid et al., 1971); bacteria (August et al.,

1962; Gottesman et al., 1962; Hardy & Kurland, 1966a,b; Payne & Boezi, 1970) and yeast (Twu & Bretthauer, 1971); (iii) poly(A) or an A-rich polyribonucleotide (see, e.g., Edmonds & Abrams, 1962, 1963; Hadjivassiliou & Brawerman, 1966; Lim et al., 1969; Edmonds & Caramela, 1969) acting as a storage form of adenine nucleotides, as suggested by Edmonds & Abrams (1962); (iv) polynucleotide phosphorylase; (v) ADP; and (vi) the respiratory chain. ATP-polymerase and poly(A)-rich polynucleotides have not yet been detected in mitochondria, although it has been claimed (Swanson, 1971) that intact mitochondria will incorporate polynucleotides, so a systematic search for the polymerase and the polymers in the isolated organelles would constitute a preliminary and comparatively simple test of this hypothesis.

Polynucleotide phosphorylase is found to be widely distributed in bacteria (Gunberg-Manago, 1963) and its presence mainly, or perhaps exclusively, in the mitochondria of animal cells is interesting. There is considerable evidence indicating that mitochondria and bacteria have very similar properties and, it had been suggested that mitochondria may have evolved from endosymbiotic bacteria (Roodyn and Wilkie, 1968; Nass, 1969; Sala and Kuntzel, 1970). The partially purified rat liver mitochondrial polynucleotide phosphorylase resembled the E. coli Q13 and PR7 mutants' polynucleotide phosphorylase which lack polymerization and exchange activities (see p. 22). From this, it may be suggested that if mitochondria evolved from bacteria, it must have mutated during evolution and lost the polymerization activity. The polymerization activity is generally accepted to be non-

functional in the bacterial cells. The role of the enzyme *in vivo* is degradative. Therefore, it would be interesting to study polynucleotide phosphorylase from lower animals and from plants. It may be possible that at certain area of the phylogenic (evolutionary) tree, the mitochondrial polynucleotide phosphorylases have both polymerization and phosphorolysis reactions.

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