Partial Purification and Properties of Rat Liver Mitochondrial Polynucleotide Phosphorylase

By YEOW PHEW SEE and P. S. FITT

Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1N 6N5, Canada

(Received 22 May 1972)

1. Polynucleotide phosphorylase was partially purified from the inner membrane of rat liver mitochondria. 2. The partially purified particulate enzyme catalyses phosphorolysis of poly(A), poly(C), poly(U) and RNA to nucleoside diphosphates. 3. It is devoid of nucleoside diphosphate-polymerization activity. 4. Variable amounts of ADP/P_i-exchange activity are associated with the polynucleotide phosphorylase and are probably due to a different enzyme. 5. ADP is the preferred substrate for exchange, and little or no reaction occurs with other nucleoside diphosphates, but ATP/P_i-exchange takes place at one-third the rate observed with ADP. 6. The partially purified enzyme is free from the phosphatases found in the crude mitochondrial inner membrane, but is associated with an endonuclease activity and some adenylate kinase activity; no cytidylate kinase activity analogous to the latter was detectable.

We have shown (See & Fitt, 1971) that rat liver mitochondria contain a polynucleotide phosphorylase that catalyses the phosphorolysis of poly(A) and poly(C) by [³²P]orthophosphate to [³²P]ADP and [³²P]CDP respectively. Nucleoside diphosphates were virtually the only radioactive products of the reaction and no significant formation of labelled nucleoside diphosphates occurred in the absence of polyribonucleotides or when the latter were replaced by nucleoside monophosphates or triphosphates. Detailed studies showed that mitochondrial polynucleotide phosphorylase is not associated with lysosomes or peroxisomes and that it is located exclusively in the mitochondrial inner membrane.

The present paper describes the partial purification and enzymic properties of polynucleotide phosphorylase from the inner membrane of rat liver mitochondria. In the accompanying paper (See & Fitt, 1972*b*), we present evidence that (i) mitochondrial polynucleotide phosphorylase is found in several animal species and in a variety of tissues of the rat and (ii) animal polynucleotide phosphorylase is present only in mitochondria and, possibly, the cell nucleus.

Experimental

Materials

Animals and chemicals were purchased from the following suppliers: radioactive substrates, Amer-sham/Searle Corp., Don Mills, Ont., Canada; Sprague-Dawley rats, Bio Breeding Laboratories, Ottawa, Ont., Canada; Triton X-100, Hartman-Leddon Co., Philadelphia, Pa., U.S.A.; digitonin,

Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; *Torula* yeast RNA (grade VI) and bovine serum albumin, Sigma Chemical Co., St. Louis, Mo., U.S.A. The sources of other materials have been described (See & Fitt, 1970).

Buffers. The pH of buffers refers to the value at 25°C.

Methods

Isolation of mitochondria and mitochondrial inner membranes. Rat liver mitochondria were isolated by the method of Schnaitman & Greenawalt (1968) with minor modifications. Six male rats (350–450 g) were decapitated and bled. The livers were removed and chilled in 100 ml of ice-cold 0.25 M-sucrose–1 mM-2-mercaptoethanol–10 mM-tris–HCl buffer, pH 7.4 ('sucrose–tris buffer'). The tissue was weighed, cut into small pieces and passed through a coarse sieve (Tissue Press; Harvard Apparatus Co., Millis, Mass., U.S.A.) to remove connective tissue. It was then homogenized in sucrose–tris buffer (2 vol.) with a motor-driven Potter–Elvehjem homogenizer (five strokes). The homogenate was diluted to 5 vol. with sucrose–tris buffer and nuclei and intact cells were removed by centrifugation at 750g for 10 min. The mitochondria were then sedimented at 10000g for 10 min and washed twice with sucrose–tris buffer (0.5 and 0.25 vol., respectively) by resuspension and centrifugation.

The mitochondrial inner-membrane-matrix fraction was prepared by treatment of the mitochondria with digitonin (Schnaitman *et al.*, 1967; Schnaitman & Greenawalt, 1968) and the inner membranes were

isolated by a modification (See & Fitt, 1971) of the method of Beattie (1969). The inner-membrane-matrix fraction obtained by differential centrifugation was resuspended in sucrose-tris buffer (14ml) and the suspension was divided into two equal portions. The samples were sonicated for 30s with a Bronwill Biosonik III sonic disintegrator (small probe; dial setting 20) and were then centrifuged for 30min at 78000g (Beckman type 30 rotor; 30000rev./min). The pellet was rinsed gently with sucrose-tris buffer (2ml). It was then transferred to a hand homogenizer with a small volume (0.1ml/g wet wt. of liver) of sucrose-tris buffer and homogenized to a fine suspension. Sufficient sucrose-tris buffer was added to give a final volume of 0.15ml/g wet wt. of liver and the inner-membrane suspension was either stored at -20°C or used immediately for purification of the enzyme (the latter 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 0.20–0.25 ml of sucrose-tris buffer/g wet wt. of liver (final protein concentration 4–6mg/ml).

Purification of mitochondrial polynucleotide phosphorylase. The frozen inner-membrane suspension (see above) was thawed and diluted with an equal volume of cold (4°C) 10% (v/v) Triton X-100–1mM-2-mercaptoethanol–10mM-tris–HCl buffer, pH7.4. The suspension was stirred for 20min and then centrifuged for 30min in a Beckman type 30 rotor at 30000rev./min. The pellet was discarded and 10mg of *Torula* yeast RNA/ml in water was added to the supernatant to a final RNA concentration of 2mg/ml. Manganese-RNA gel ('Mn-RNA' gel) (1mg of RNA/g wet wt. of liver; for preparation, see below) was added and the suspension was stirred at 4°C for 1h. The gel was collected by centrifugation and the supernatant was treated as described above with Mn-RNA gel. The combined Mn-RNA-gel pellets were washed with sucrose-tris buffer (10ml) by suspension in a Dounce homogenizer with a 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, pH7.4 ('tris-ME buffer') and applied at 60ml/h to a column (1.7cm $\times$ 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 (about 3cm thick) of DEAE-cellulose. 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 solid $(\text{NH}_4)_2\text{SO}_4$ (472g/litre) was added. The suspension was stirred for 20min at 4°C

and the precipitate (not visible owing to large volume) was collected by centrifugation at 16000g for 15min. The very-small pellet was dissolved in tris-ME buffer (50 μ l/g wet wt. of liver) to give a slightly cloudy yellow solution, which was dialysed overnight against tris-ME buffer (2litres). During the dialysis, the enzyme aggregated to give a fine white precipitate. The suspension was stored at 4°C (freezing and thawing causes a 30–40% loss of enzymic activity at this stage).

Preparation of manganese-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 concentrated NH_3 and the mixture was stirred until all the RNA dissolved. The volume was adjusted with water to give a final RNA concentration of 10mg/ml. Then 1M-MnCl₂ (2vol.) was added and stirring was continued for a further 10min. 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. The gel was then suspended in the same volume of cold (4°C) 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.

Enzyme assays. All incubations were performed in a water-bath shaker.

(a) Phosphorolysis activity of mitochondrial polynucleotide phosphorylase was measured by the method of See & Fitt (1970). The standard assay medium (0.1ml) contained: 0.1M-tris-HCl buffer, pH8.2 (or 7.8, if used for the membrane-associated enzyme); 6mM-MgCl₂; 1mM-2-mercaptoethanol; 10mM-K₂H³²PO₄ (50000–100000c.p.m./ μ mol); poly(A), 1.5mg/ml; 0.1% (v/v) Triton X-100; and enzyme as appropriate. Incubation was at 37°C for 1h. The reaction was stopped with a charcoal-HClO₄ suspension and the charcoal was washed, dried and counted for radioactivity as previously described (See & Fitt, 1970). Controls lacking enzyme or poly(A) were performed in all cases and the results corrected accordingly. A unit was defined as the amount of enzyme catalysing incorporation of 1nmol of [³²P]P_i/h into charcoal-adsorbable material.

(b) NDP/P_i-exchange activity. The reaction mixture (0.1ml) contained: 0.1M-tris-HCl buffer, pH7.8; 6mM-MgCl₂; 1mM-2-mercaptoethanol; 10mM-K₂H³²PO₄ (50000–100000c.p.m./ μ mol); 5mM-NDP; 0.1% (v/v) Triton X-100; and enzyme as appropriate. Incubation was at 37°C for 1h and the samples were processed as described for the phosphorolysis assay. Controls lacking enzyme or NDP were performed in all cases and the results corrected accordingly. The amount of P_i incorporated was calculated from the expression (Grunberg-Manago *et al.*, 1956): nmol of P_i incorporated = c.p.m. incor-

porated $\times$ (nmol of P_i + nmol of NDP)/c.p.m. in reaction. One unit was defined as the amount of enzyme catalysing incorporation of 1 nmol of P_i /h into charcoal-adsorbable material.

(c) The activity of nucleases capable of degrading poly(A) was determined by measuring the release of acid-soluble radioactivity during hydrolysis of ^{14}C -labelled poly(A). 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 -labelled poly(A), 1.5 mg/ml (36000 c.p.m./mg); 0.1% (v/v) Triton X-100; and enzyme as appropriate. Incubation was at 37°C for 1 h and the reaction was stopped by addition of 7% (w/v) HClO_4 (0.1 ml). The mixture was kept in ice for 10 min and then centrifuged in a clinical centrifuge at room temperature. Samples (50 μl) of the supernatant were dried on planchets and counted for radioactivity in a Nuclear-Chicago low-background thin-window counter. Controls lacking enzyme were performed in a similar way and their radioactivity was subtracted from the assay values.

(d) Pyrophosphatase and phosphatase activities were assayed by the following method. The reaction mixture (1 ml) contained: 0.1 M-tris-HCl buffer, pH 8.0; 1 mM-nucleoside diphosphate (or other substrates, as indicated); 5 mM- MgCl_2 ; 1 mM-2-mercaptoethanol; 0.1% (v/v) Triton X-100; and enzyme as appropriate. Incubation was at 37°C for 30 min in a water-bath shaker. The reaction was stopped with cold 6% (w/v) HClO_4 (1 ml) and the P_i released was determined by the method described below. Controls lacking enzyme were performed in all cases and the results corrected accordingly. A unit of phosphatase activity was defined as the amount of enzyme catalysing the formation of 1 μmol of P_i /h.

Orthophosphate assay. P_i was determined by the following method, developed (See & Fitt, 1972a) for use in the presence of Triton X-100. The following stock reagents were used: (A) 2% (v/v) Triton

X-100 in 1 M-sodium acetate buffer, pH 4.0; (B) 1% (w/v) ammonium molybdate tetrahydrate in 0.025 M- H_2SO_4 (stored in plastic bottles in the dark); (C) sodium metabisulphite (15 g) and *p*-methylaminophenol sulphate (5 g) in water (500 ml). The deproteinized test sample (1 ml) containing 3% (w/v) HClO_4 and 0.05–1.0 μmol of P_i was mixed with 1 M-sodium acetate (0.7 ml) and water (0.3 ml). Reagents A, B and C (1 ml of each) were added in the indicated order and the mixtures were kept at room temperature for 15 min. Their E_{650} was then determined against a control lacking P_i , in 1 cm path-length cells.

Protein assay. Protein was determined by the method of Lowry *et al.* (1951) in the presence of sodium dodecyl sulphate as previously described (See & Fitt, 1970), with bovine serum albumin as standard.

Phospholipids. Phospholipids were characterized as previously described (See & Fitt, 1970).

Results and Discussion

Purification of rat liver mitochondrial polynucleotide phosphorylase

The method of purification of polynucleotide phosphorylase from rat liver mitochondria described in the Experimental 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 measured as well as that of the phosphorylytic activity, are shown in Table 1.

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 $(\text{NH}_4)_2\text{SO}_4$ concentration was only slightly cloudy, but large particles formed during the final dialysis against the low-ionic-strength tris-ME buffer. Addition of Triton

Table 1. *Purification of polynucleotide phosphorylase from rat liver mitochondria*

The enzyme was purified as described in the Experimental section and poly(A) phosphorolysis and ADP/ P_i -exchange activities were determined by their respective standard assays [i.e. in the presence of 0.1% (v/v) Triton X-100].

Preparation	Fraction	Phosphorolysis activity		Exchange activity	
		(units)	(units/mg)	(units)	(units/mg)
1	Whole mitochondria	54 120	191	91 896	324
	Inner membrane	44 776	274	70 222	431
	Purified enzyme	24 539	1624	4400	291
2	Whole mitochondria	30 203	120	50 641	201
	Inner membrane	28 501	182	46 592	297
	Purified enzyme	18 385	1332	8233	581
3	Whole mitochondria	—	—	—	—
	Inner membrane	38 931	113	82 624	240
	Purified enzyme	10 008	1713	0	0

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 78000g for 30min 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 0.25M-NaCl followed by dialysis against the buffer at low ionic strength. A similar tendency to aggregate as a result of changes in the ionic strength of the medium has been observed with erythrocyte-membrane proteins (Mazia & Ruby, 1968; Blumenfeld *et al.*, 1970), ox heart mitochondrial cytochrome oxidase (Sun *et al.*, 1968), hydrophobic lipoproteins from *Neurospora crassa* and *Hydrogenomonas facilis* (Kuehn *et al.*, 1969; Shannon & Hill, 1971) and guinea-pig liver polynucleotide phosphorylase (See & Fitt, 1970).

The particulate partially purified mitochondrial enzyme usually contained small amounts of phospholipids, mainly cardiolipin and phosphatidylethanolamine, but the total and relative amounts of the lipids varied from one preparation to another and they were undetectable on one occasion. Therefore the polynucleotide phosphorylase does not require lipids for activity, although it is membrane-associated. This conclusion is supported by our observation that extraction of intact mitochondria with either 96% (v/v) acetone, which removes all the neutral lipids (Lester, 1967), or 90% (v/v) acetone, which removes 80% of total phospholipids (Lester, 1967), did not affect the phosphorolytic activity. These treatments are known (Fleischer & Fleischer, 1967) to inactivate enzymes of the electron-transport chain, which can then be reactivated by recombination with the lipids. However, the acetone treatments did render mitochondrial polynucleotide phosphorylase insoluble in buffer containing 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 phosphorolytic activity, the lower the exchange activity detected and, in one case (Table 1, preparation 3), the preparation was completely free of exchange activity. These observations support the conclusion (see below) that the polynucleotide phosphorylase and exchange activities are due to different enzymes.

When stored at 4°C, 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 suspension was frozen, and thawed after overnight storage at -20°C, it lost 30-40% of its activity.

Enzymic properties of mitochondrial polynucleotide phosphorylase

The evidence that the enzyme is both a true polynucleotide phosphorylase and that it is located exclusively in the mitochondrial inner membrane has been published (See & Fitt, 1971).

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. 1) 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 below), which had a pH optimum of 8 and was entirely eliminated during the

Fig. 1. Dependence of phosphorolysis and exchange activities on pH

The poly(A) phosphorolysis and ADP/P_i-exchange activities were measured in the standard assay conditions (see the Experimental section) with buffers of the indicated pH: ●, phosphorolysis, purified enzyme; ○, phosphorolysis, crude inner-membrane suspension; ▲, exchange, purified enzyme.

Fig. 2. Influence of bivalent cations on the phosphorolysis and exchange activities of rat liver mitochondrial polynucleotide phosphorylase

The partially purified enzyme was assayed (a) for poly(A) phosphorolysis activity and (b) for ADP/P₁-exchange activity by the appropriate standard assay (see the Experimental section) with the indicated concentrations of MgCl₂ (●), MnCl₂ (○) and CaCl₂ (▲).

Table 2. Substrate specificity of the phosphorolysis activity of mitochondrial polynucleotide phosphorylase

Phosphorolysis activity was measured by the standard assay (see the Experimental section) 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 (approx. 99 c.p.m.) were subtracted from the appropriate assay values. The enzyme fractions were from preparation no. 1 (Table 1).

Substrate	^{[32]P} P ₁ incorporation (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

RNA-gel-DEAE-cellulose step of the purification procedure.

The enzyme required Mg²⁺ for activity (Fig. 2a), with an optimum concentration of 6 mM. Mn²⁺ could partially replace Mg²⁺, but no phosphorolysis of poly(A) occurred in the presence of Ca²⁺.

The poly(A)-saturation curve of partially purified mitochondrial polynucleotide phosphorylase reached a plateau at 1 mg/ml. Table 2 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-molecular-weight wheat-germ RNA to some extent. Little or no reaction occurred with poly(G), and DNA was not a substrate. These results are in general agreement with those previously reported for both bacterial (Grunberg-Manago, 1963) and animal (See & Fitt, 1970) polynucleotide phosphorylase. 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). This apparent difference was, once again, due to the phosphatases that are removed during the

Table 3. *Substrate specificity of inner-membrane phosphatases from rat liver mitochondria*

Phosphatase activity of the mitochondrial inner-membrane suspension (Table 1, preparation no. 2) was measured by the procedure described in the Experimental section with and without Mg^{2+} , by using 0.5mg of membrane-suspension protein/ml. The result with PP_i as substrate is not corrected for the release of two equivalents of P_i /bond cleaved.

Substrate	P _i released (μ mol/30min)	
	+Mg ²⁺	-Mg ²⁺
ATP	1.08	1.07
ADP	0.40	0.49
AMP	0.43	0.23
CDP	0.09	0.02
GDP	0.18	0.01
UTP	0.44	0.03
UDP	0.20	0.03
UMP	0.53	0.30
PP _i	1.53	0.01
<i>p</i> -Nitrophenyl phosphate	0.03	0.02

purification and that are much less active with CDP than with either ADP or UDP.

The results in Table 3 show that inorganic pyrophosphate and a wide variety of ribonucleotides were readily hydrolysed by the mitochondrial inner-membrane phosphatases in the presence of Mg^{2+} . In the absence of Mg^{2+} , only ADP and ATP were hydrolysed at an undiminished rate. However, addition of EDTA (1mM) to the reaction medium, or prior exposure of the membranes to 10mM-EDTA in 1mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 7.4, followed by dialysis against buffer lacking the chelating agent, caused an 80-90% decrease in the rate of hydrolysis with ADP as substrate in the absence of added Mg^{2+} , indicating that bivalent cations were also required in this case. Addition of increasing amounts of either Mg^{2+} or Ca^{2+} to the assay medium containing EDTA restored the activity with ADP progressively to its original value. The phosphatase(s) had a pH optimum of 8 and were not non-specific, because *p*-nitrophenyl phosphate was not hydrolysed significantly.

Fig. 3 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, so that the measured activity de-

Fig. 3. *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 suspension and the partially purified enzyme were measured in the standard assay conditions (see the Experimental section) with and without 0.1% (v/v) Triton X-100, as indicated: ■, partially purified enzyme (2.9mg of protein/ml), with Triton X-100; ●, inner-membrane suspension (5.87mg of protein/ml), with Triton X-100; ○, inner-membrane suspension (1:2 dilution), with Triton X-100; ▲, inner-membrane suspension (5.87mg of protein/ml), without Triton X-100.

creased rapidly above 40 units/0.1 ml (about 200 μ g of protein/0.1 ml), but 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. However, the detergent stimulated phosphorolysis by the inner-membrane suspension by 100% and by the partially purified enzyme by 30%.

There was a lag phase in the reaction catalysed by either the crude or the 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 min.

Since digitonin is used in the preparation of the inner mitochondrial membranes, its effect on the phosphorolytic activity of the enzyme was studied. Only a slight inhibition was observed with digitonin concentrations below 0.1mM and some stimulation occurred at higher concentrations. Dialysis of the

Fig. 4. 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-MgCl₂; 1 mM-2-mercaptoethanol; 5 mM-[¹⁴C]ADP (659000 c.p.m./μmol); 0.1% (v/v) Triton X-100; 1.6 mM-ApA, where appropriate; and partially purified particulate polynucleotide phosphorylase from rat liver mitochondria, 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 left 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 supernatants 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 h by descending chromatography with 1 M-ammonium acetate buffer (pH 7.1)–95% (v/v) ethanol (7:3, 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 by liquid-scintillation counting. ●, Control; ○, reaction mixture with ApA (the results without ApA were virtually superimposable, and are therefore not shown).

washed inner membranes had no significant effect on their polynucleotide phosphorylase activity.

All attempts to detect any nucleoside diphosphate polymerization activity associated with the partially purified enzyme failed. Many combinations of the following parameters were tried by using standard incorporation assay techniques (Grunberg-Manago, 1963; Gajda *et al.*, 1970) that would detect the forma-

tion of acid-insoluble radioactive products from ¹⁴C-labelled nucleoside diphosphates: (i) 1–5 mM-ADP, 5 mM-CDP, 5 mM-UDP; (ii) 1–8 mM-MgCl₂ and 0.2–8 mM-MnCl₂ (cf. Babinet *et al.*, 1965); (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 (adenosine 3':5'-cyclic monophosphate) (5 μmol/ml), (c) ApApA (100 and 200 μg/ml), (d) oligo- and

poly-lysines and polyornithine (cf. Fitt & Wille, 1969), (e) 10mM-NaCl, (f) Triton X-100 or cetyltrimethylammonium bromide. Since such methods would fail to detect a very limited incorporation into an acid-soluble oligonucleotide, a search for such a product was made. The experiment described in Fig. 4 shows that no detectable formation of oligonucleotides occurs with mitochondrial polynucleotide phosphorylase in the presence or the absence of ApA, in conditions that should have been appropriate if the ADP/P_i-exchange activity of the preparation (see below) depended on continued formation and phosphorolysis of a short oligonucleotide, in accordance with the mechanism considered to be responsible for the exchange activity of the bacterial enzymes (Grunberg-Manago, 1963; Bon *et al.*, 1970; Chou & Singer, 1971).

The enzyme contained a nuclease that degraded ¹⁴C-labelled poly(A) to acid-soluble products, but chromatographic analysis of the latter (Fig. 5) showed that they were almost entirely oligonucleo-

tides: little or no AMP (which would not have separated from the ADP used as substrate in the experiment described in Fig. 4) was formed. Terminally phosphorylated oligonucleotides were not available for use as standards in these experiments, but the use of the series (Ap)_nA gave an indication of the size of the products and was justified by the failure of the mononucleotides to separate according to their degree of phosphorylation in the chromatographic system used. Thus the failure to detect oligonucleotides in the experiment described in Fig. 4 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 *Escherichia coli* Q13 polynucleotide phosphorylase, which lacks (Thang *et al.*, 1969) incorporation and NDP/P_i-exchange activities.

The endonuclease activity present in the partially purified mitochondrial polynucleotide phosphorylase

Fig. 5. Chromatographic analysis of the products of degradation of ¹⁴C-labelled poly(A) by nucleases present as contaminants in mitochondrial polynucleotide phosphorylase

The reaction mixture (0.1 ml) contained: 0.1 M-tris-HCl buffer, pH 7.8; 6 mM-MgCl₂; 1 mM-2-mercaptoethanol; ¹⁴C-labelled poly(A), 1.5 mg/ml (168 000 c.p.m./mg); 0.1 % (v/v) Triton X-100; and partially purified (particulate) mitochondrial polynucleotide phosphorylase, 100 units of phosphorolysis activity. The control was a similar mixture lacking enzyme. Incubation, chromatography (for 16 h) and subsequent operations were performed as described in Fig. 4. ●, Control; ○, reaction mixture.

may be the enzyme active with poly(A) that was described by Morais (1969): chromatographic analysis of the products obtained by hydrolysis of poly(A) in the conditions known (Morais *et al.*, 1967) to be optimum for this endonuclease gave the same distribution of products, but a 20% increase in overall reaction. Although most of the activity was associated with the outer membrane of mitochondria fractionated by the digitonin method (Morais, 1969), about 12% of the enzyme was still found in the inner-membrane-matrix fraction and could thus be responsible for the endonuclease activity we have observed.

The partially purified polynucleotide phosphorylase was also contaminated by some adenylate kinase, in agreement with the reports of Schnaitman & Pedersen (1968) and Kopaczyk *et al.* (1968) that part of the adenylate kinase activity of mitochondria was associated with the inner membrane of the organelle. However, no cytidylate kinase activity capable of converting CDP into CMP and CTP was present in the inner-membrane-associated enzyme, thus eliminating the possibility that such enzymes could significantly affect the results of the phosphorolysis experiments.

Nucleoside diphosphate/orthophosphate-exchange activity

The partially purified polynucleotide phosphorylase was associated with a highly specific P_i -exchange activity (Table 4 and Fig. 6). ADP was the preferred substrate, but exchange also occurred with ATP at 31% of the rate observed with the diphos-

phate (Table 4). The ATP used was shown to contain <0.9% (mol/mol) of ADP by t.l.c. (Randerath, 1964) followed by elution and spectrophotometric determination of the separated nucleotides. GDP and UDP were not substrates, but a very limited activity was observed with CDP (Table 4) with the enzyme from one preparation (Table 1, preparation 2). (In this latter experiment, three times as many ADP/ P_i -exchange units were used as in the experiments described in Fig. 6, in which no detectable reaction occurred with CDP.) The results in Fig. 6 show that the specificity for ADP was not due to different substrate-concentration optima in the case of the other diphosphates.

In the conditions of the standard ADP/ P_i -exchange assay, the enzyme-saturation curve was linear at low enzyme concentrations, unlike the case of the phosphorolytic activity (see above), and incorporation

Fig. 6. *Substrate specificity of the exchange activity associated with rat liver mitochondrial polynucleotide phosphorylase*

Table 4. *Substrate specificity of exchange activity*

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

Substrate	$[^{32}P]P_i$ incorporated (c.p.m.)	
	120 $\pm$ 5	—Control
None (control; six determinations)	120 $\pm$ 5	—
ATP	1148	1028
ADP	3328	3208
AMP	93	0
CDP	240	120
GDP	116	0
UDP	129	9

The NDP/ P_i -exchange activity of the partially purified enzyme (Table 1, preparation no. 1) was measured in the conditions of the standard exchange assay (see the Experimental section) at the indicated concentrations of the nucleoside diphosphates and with 17 units of ADP/ P_i -exchange activity/assay. The controls 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).

of P_i was linear for 90 min with up to 50 units of ADP/ P_i -exchange activity.

Since the exchange and phosphorolysis reactions were inhibited 40% by the addition of ApA (2 mg/ml; 3.2 mM) to the appropriate assay medium, and the preparation did not catalyse ADP polymerization (see above), it appears that the exchange reaction cannot depend 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 NDP/ P_i exchange, and oligonucleotides with free 3'-hydroxyl groups stimulate the reaction (Singer & Guss, 1962; Bon *et al.*, 1970; Chou & Singer, 1971).

The two activities were also inhibited by NaCl (Fig. 7), although the exchange reaction was somewhat less sensitive to changes in the ionic strength of the medium, and they had very similar stabilities at both 4°C and 37°C. These observations, together with the similarity of the effect of ApA on the two reactions, suggest that both activities might 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 2), 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 preparation (Table 1, preparation 3) was devoid 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%, whereas 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, whereas the lower concentration did not affect the ADP/ P_i -exchange activity and the higher one caused only a 10% inhibition.

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.

We thank the Medical Research Council of Canada for an operating grant (to P. S. F.) and the Province of Ontario for a Graduate Fellowship (to Y. P. S.).

Fig. 7. Effect of NaCl on the phosphorolysis and exchange activities of rat liver mitochondrial polynucleotide phosphorylase

The activity of the partially purified enzyme (Table 1, preparation no. 1) was determined in the standard poly(A) phosphorolysis and ADP/ P_i -exchange assays at the indicated NaCl concentrations. ●, Phosphorolysis (40 units); ○, exchange (17 units).

References

- Babinet, C., Roller, A., Dubert, J. M., Thang, M. N. & Grunberg-Manago, M. (1965) *Biochem. Biophys. Res. Commun.* **19**, 95-101
- Beattie, D. S. (1969) *Biochem. Biophys. Res. Commun.* **35**, 67-74
- Blumenfeld, O. O., Gallop, P. M., Howe, C. & Lee, J. T. (1970) *Biochim. Biophys. Acta* **211**, 109-123
- Bon, S., Godefroy, T. & Grunberg-Manago, M. (1970) *Eur. J. Biochem.* **16**, 363-372
- Chou, J. Y. & Singer, M. F. (1971) *J. Biol. Chem.* **246**, 7486-7496
- Fitt, P. S. & Wille, H. (1969) *Biochem. J.* **112**, 497-503
- Fleischer, S. & Fleischer, B. (1967) *Methods Enzymol.* **10**, 406-433
- Gajda, A. T., Zaror de Behrens, G. & Fitt, P. S. (1970) *Biochem. J.* **120**, 753-761
- Grunberg-Manago, M. (1963) *Progr. Nucl. Acid Res.* **1**, 93-133
- Grunberg-Manago, M., Ortiz, P. J. & Ochoa, S. (1956) *Biochim. Biophys. Acta* **20**, 269-285
- Kopaczky, K., Asai, J., Allman, D. W., Oda, T. & Green, D. E. (1968) *Arch. Biochem. Biophys.* **123**, 602-621

- Kuehn, G. D., McFadden, B. A., Johanson, R. A., Hill, J. M. & Shumway, L. K. (1969) *Proc. Nat. Acad. Sci. U.S.* **62**, 407-414
- Lester, R. L. (1967) *Methods Enzymol.* **10**, 264-266
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Mazia, D. & Ruby, A. (1968) *Proc. Nat. Acad. Sci. U.S.* **61**, 1005-1012
- Morais, R. (1969) *Biochim. Biophys. Acta* **189**, 38-45
- Morais, R., Blackstein, M. & de Lamirande, G. (1967) *Arch. Biochem. Biophys.* **121**, 711-716
- Randerath, K. (1964) *Thin Layer Chromatography*, p. 192, Verlag Chemie, Weinheim
- Schnaitman, C. & Greenawalt, J. W. (1968) *J. Cell Biol.* **38**, 158-175
- Schnaitman, C. A. & Pedersen, P. L. (1968) *Biochem. Biophys. Res. Commun.* **30**, 428-433
- Schnaitman, C., Erwin, V. G. & Greenawalt, J. W. (1967) *J. Cell Biol.* **32**, 719-735
- See, Y. P. & Fitt, P. S. (1970) *Biochem. J.* **119**, 517-524
- See, Y. P. & Fitt, P. S. (1971) *FEBS Lett.* **15**, 65-67
- See, Y. P. & Fitt, P. S. (1972a) *Anal. Biochem.* in the press
- See, Y. P. & Fitt, P. S. (1972b) *Biochem. J.* **130**, 355-362
- Shannon, A. J. & Hill, J. M. (1971) *Biochemistry* **10**, 3021-3029
- Singer, M. F. & Guss, J. K. (1962) *J. Biol. Chem.* **237**, 182-189
- Sun, F. F., Prezbindowski, K. S., Crane, J. L. & Jacobs, E. E. (1968) *Biochim. Biophys. Acta* **153**, 804-818
- Thach, R. E. (1966) *Procedures Nucleic Acid Res.* **1**, 520-534
- Thang, M. N., Thang, D. C. & Grunberg-Manago, M. (1969) *Eur. J. Biochem.* **8**, 577-590