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Preparation and General Properties of a Soluble Adenosine Triphosphatase from Mitochondria

By M. J. SELWYN*
Department of Biochemistry, University of Cambridge

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1. The purification of an adenosine triphosphatase present in aqueous extracts of acetone-dried ox-heart mitochondria is described. 2. No evidence was found for the presence of more than one protein having adenosine-triphosphatase activity in these extracts. 3. The enzyme is less stable at 0° than at 25° but is stabilized by glycerol. 4. The activity is dependent on the presence of Mg^{2+} or certain other bivalent metal cations. 5. The adenosine-triphosphatase activity of the Mg^{2+}-activated enzyme is enhanced by 2,4-dinitrophenol. 6. The kinetics of Mg^{2+} activation indicate that the ATP–Mg^{2+} complex is the important substrate: free ATP and Mg^{2+} are inhibitory. 7. This preparation of mitochondrial adenosine triphosphatase has many properties in common with the adenosine triphosphatase coupling factor from mitochondria (Racker, 1961).

Soluble ATPase† activity was found in aqueous extracts of acetone-dried rat-liver mitochondria by Lardy & Wellman (1953), but no purification of the enzyme was reported. This ATPase activity was stimulated by 2,4-dinitrophenol, and Lardy & Wellman (1953) postulated that this provided a basis for the action of 2,4-dinitrophenol on whole mitochondria.

A Mg^{2+}-requiring 2,4-dinitrophenol-stimulated ATPase was obtained in solution by Pullman, Penefsky, Datta & Racker (1960) by disrupting mitochondria in a Nossal shaker, and this ATPase was shown to be a necessary factor for restoring the coupling of the phosphorylation of ADP to electron transport by the submitochondrial particles obtained in the same treatment (Penefsky, Pullman, Datta & Racker, 1960). This ATPase was purified about 100-fold.

An ATPase resembling actomyosin was obtained from mitochondria by extraction with 0·6M-potassium chloride (Ohnishi & Ohnishi, 1962) and partly purified, but its properties have not been sufficiently investigated to tell whether it is the same enzyme (presumably combined with an actin-like protein) as that described by Pullman *et al.* (1960).

Investigators of the ATPase activity of whole mitochondria or submitochondrial particles have postulated the existence of up to four different ATPases in mitochondria. These hypotheses, however, have been based on indirect evidence such as multiple peaks or shoulders in curves showing the effect of pH on ATPase activity in the presence and absence of 2,4-dinitrophenol (Myers & Slater, 1958) or arsenate (Wadkins, 1961) or differential sensitivity to inhibitors (Hemker, 1963).

Penniall (1960) has claimed that crude extracts of acetone-dried mitochondria contain several ATPases, and Beyer (1960) has observed release of two ATPases on prolonged ultrasonic treatment of mitochondria.

There are alternative explanations for these observations, which are discussed below in relation to the results described in the present paper, and there is at present no consensus of opinion about the number of ATPases present in mitochondria.

The present work was undertaken with the objects of investigating the mode of action of 2,4-dinitrophenol on the soluble ATPase present in extracts of acetone-dried mitochondria, investigating the relation between this ATPase and those reported by other workers and attempting to obtain conclusive evidence for the presence of more than one ATPase in mitochondria.

The present paper describes the purification and general properties of the ATPase obtained from acetone-dried ox-heart mitochondria. A preliminary account of this work has been published (Selwyn, 1962).

EXPERIMENTAL

Materials

ATP (disodium salt), ADP (sodium salt), AMP (sodium salt), ITP (sodium salt), *p*-nitrophenyl phosphate (sodium salt) and tris (Sigma 121 or Trizma base) were obtained

* Present address: School of Biological Sciences, University of East Anglia, Norwich, NOR 77X.

† Abbreviation: ATPase, adenosine triphosphatase.

from Sigma Chemical Co., St Louis, Mo., U.S.A. Pentasodium tripolyphosphate was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, as pentasodium triphosphate and purified by the method of Watters, Loughran & Lambert (1956). 2,4-Dinitrophenol and *p*-nitrophenol were obtained from British Drug Houses Ltd., Poole, Dorset, and purified by recrystallization from water. Ribose 5-phosphate was obtained from L. Light and Co., Colnbrook, Bucks., dissolved in dilute H_2SO_4 and neutralized with NaOH. DEAE-cellulose powder (Whatman DE-50) was obtained from H. Reeve Angell, London, E.C.4. Sephadex dextran gel was obtained from Pharmacia, Uppsala, Sweden. Partially hydrolysed starch was obtained from the Connaught Medical Research Laboratories, Toronto, Canada.

Other reagents were of A.R. or highest available grade and were obtained from British Drug Houses Ltd. or Hopkin and Williams Ltd.

All reagents were made up in glass-distilled water.

The pH of buffer solutions was checked with an E.I.L. pH-meter standardized with solutions prepared from standard buffer tablets (Burroughs, Wellcome and Co., London, N.W. 1).

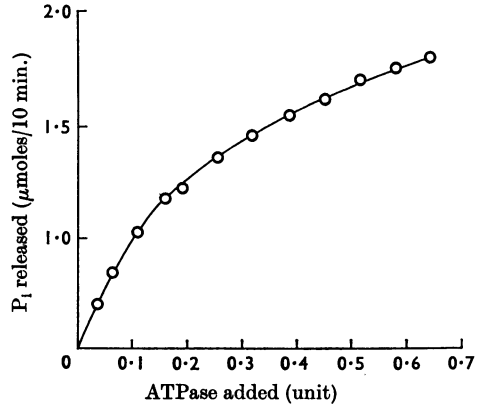

Fig. 1. Calibration curve of standard assay. Different volumes of a solution of purified ATPase (specific activity 15 units/mg. of protein) were assayed by the standard procedure for 10 min. at 25°. The scale of the abscissa has been adjusted to read directly in units of ATPase activity.

Methods

ATPase assay. The enzyme was assayed in extraction, purification and inactivation experiments by measuring the amount of P_i liberated in a given time in 2·5 mM-ATP–2·5 mM-$MgSO_4$–50 mM-tris–HCl buffer, pH 7·4, in a final volume of 2·0 ml. at 25°. Phosphate was estimated by the method of Fiske & Subbarow (1925). The reaction was started by adding the enzyme sample and stopped by adding 2 ml. of 10% (w/v) trichloroacetic acid or 1·0 ml. of 2·5% (w/v) ammonium molybdate in 5 N-H_2SO_4 or the reagents for the phosphate estimation mixed and diluted to 8·0 ml. (the ammonium molybdate–H_2SO_4 reagent must be diluted before adding the solution of the reducing agent and the mixture must be prepared each day). The second and third methods were only used with partially purified enzyme when the protein blank had E_{660} 0·005 or less in the phosphate assay.

A unit of ATPase is defined as the amount of enzyme that liberates 1 μmole of P_i from ATP in 1 min. under the standard assay conditions.

Since the progress curve of the reaction is not linear (Selwyn, 1965) the amount of P_i released in a given time is not proportional to the amount of enzyme added and a calibration curve (Fig. 1) was constructed for converting μmoles of P_i released into units of enzyme activity. The usable range of this curve is 0·05–0·65 unit of ATPase, but below 0·05 unit the curve can be assumed to be linear and the amount of enzyme found by linear interpolation. Under fixed conditions the amount of P_i produced is a function of the product of time of incubation and the amount of enzyme added (Selwyn, 1965), and hence the range of this curve can be extended by incubating for times other than 10 min. if the amount of enzyme read off the curve is multiplied by the factor $10/t$, where t is the time of incubation in minutes.

Measurement of rates. The marked curvature of the progress curves and the necessity for withdrawing samples and stopping the reaction before measuring its extent made estimation of the initial rates difficult. In the experiments quoted initial rates were estimated by using less than 0·25 unit of ATPase/10 ml. of medium and not sampling after 10 min. In this way approximately linear curves were obtained: preliminary experiments showed that there was little gain in accuracy of estimation of the initial rate if more enzyme was added or if the reaction was allowed to proceed for a longer time. To allow comparison between experiments with samples of different specific activity the rates are expressed relative to the rate measured under the standard assay conditions.

Starch-gel electrophoresis. Starch gels were prepared with partially hydrolysed starch by the method of Smithies (1955, 1959) and were poured into trays 20 cm. × 2·5 cm. × 0·5 cm. deep. The buffer used was the discontinuous system of Poulik (1957) at pH 8·6. Electrophoresis was carried out for up to 6 hr. at 200 v giving a current of 10–20 mA. After electrophoresis the gels were sliced in half horizontally; one half was stained for ATPase activity, the other for protein [with Nigrosine W.S. in methanol–water–acetic acid (5:5:1, by vol.) followed by washing in the same medium]. ATPase activity was detected by incubating the gel slice in the medium of Wachstein & Meisel (1957), namely 1 mM-ATP–10 mM-$MgSO_4$–3·5 mM-$Pb(NO_3)_2$–80 mM-tris–maleic acid buffer, pH 7·4, for 2 hr. at 37°. In some experiments the gel slice used for ATPase detection was sliced vertically and the two portions were incubated under identical conditions except that 2,4-dinitrophenol (0·5 mM) was added to the medium for one portion of the gel. After incubation the gel slices were washed in frequent changes of distilled water for about 20 min. and then developed in dilute Na_2S solution.

Protein estimation. Protein was estimated spectrophotometrically by the method of Warburg & Christian (1941).

Acetone-dried mitochondria. Mitochondria were prepared from ox-heart muscle by a method based on that of Crane, Glenn & Green (1956). All operations were performed in the cold with chilled apparatus and ice-cold media. The hearts were obtained within 1 hr. of the slaughter of the animals and were packed in ice. Tendons, fat and valves were removed and the muscle was cut into 3 cm. cubes, which were

placed in polythene bags and thoroughly cooled before passage through a mincer with 2mm. holes in the end plate. The muscle was homogenized in a Waring Blendor with 2·5l. of 0·25M-sucrose–10mM-tris–0·5mM-EDTA/kg. of muscle. During homogenization, which takes from 30sec. to 4min. depending on the make and condition of the blender, a further 1·5l. of the same medium was added. The final pH was usually about pH 7·5, but if it was markedly lower it was adjusted with 1·0M-tris. The homogenate was centrifuged at 1000*g* for 15min., and the supernatant fluid poured off through two layers of lint and centrifuged at 20000*g* for 20min. to precipitate the mitochondria. The pellet was resuspended in 400ml. (per kg. of starting material) of 0·12M-KCl–10mM-tris–HCl buffer, pH 7·4, and centrifuged at 20000*g* for 20min. The pellet from this centrifugation is suspended in enough KCl medium to allow it to be poured easily.

Up to 80ml. of mitochondrial suspension was poured in a thin stream into 800ml. of acetone at −20° in the vessel of a Waring Blendor (1l. size) running at half speed and the mixture blended at full speed for about 2min. It is essential to have a ratio of acetone to suspension of at least 10:1, for otherwise the temperature rises above −5° during blending and also the mitochondria are insufficiently dehydrated and filtration is prolonged. If the mitochondria remain in contact with wet acetone for long periods only weak ATPase activity remains.

The desiccated mitochondria were filtered off on a Buchner funnel (the pad of mitochondria should not be more than about 0·5cm. thick) and washed with several portions of acetone at −20° (a total of about 1l. of acetone for every 80ml. of mitochondrial suspension). The pad was sucked dry, broken up, pressed between layers of filter paper and placed over anhydrous $CaCl_2$ in a desiccator, which was evacuated for 1hr. at room temperature and then left overnight. The following day evacuation was repeated until there was no odour of acetone. The dry powder can be stored over anhydrous $CaCl_2$ or silica gel at −20° for several months without loss of activity.

Purification of the ATPase

All operations during extraction and purification of the ATPase were performed at room temperature.

Extraction of the ATPase. It was found to be necessary to produce a fine dispersion of the acetone-dried mitochondria but at the same time to avoid much foaming. An electrically driven all-glass Potter–Elvehjem homogenizer produced a satisfactory dispersion and for large-scale extractions it proved convenient to use a homogenizer with a large bowl (300ml. capacity) and an outlet at the lower end of the ground tube. After breaking up lumps of the powder in a conventional Potter–Elvehjem or Dounce homogenizer the suspension was poured through the flow-through homogenizer at intervals for about 1hr. A suitable suspension contained 20–25mg. of the acetone-dried mitochondria/ml. of water; this gave a convenient concentration of enzyme in the extract and the wet powder did not occlude too much of the supernatant. Before centrifugation 50mM-tris–H_2SO_4 buffer, pH 7·5, was added, as a less turbid supernatant was then obtained. The suspension was centrifuged at 20000*g* for 20min. at room temperature (a non-refrigerated Servall Superspeed centrifuge was used for most preparations). A faintly turbid yellow supernatant containing 50–80% of the ATPase activity of the whole suspension was poured off from the tightly packed precipitate. In initial experiments centrifugation at 100000*g* for 1hr. was used. This produced a supernatant that was not turbid and showed that the ATPase was soluble, but has no advantages if the enzyme is to be further purified.

First ammonium sulphate fractionation. Solid $(NH_4)_2SO_4$ was added to the extract (24·2g./100ml.) to produce a 40%-saturated solution, the solid was dissolved by careful agitation and the solution allowed to stand for 15min. before centrifugation at 20000*g* for 15min. The supernatant was carefully decanted and more solid $(NH_4)_2SO_4$ added to it to bring the solution to 50% saturation [an additional 7·0g. of $(NH_4)_2SO_4$/100ml. of original extract]. The solution was allowed to stand and centrifuged as before, and the precipitate collected and redissolved in 50mM-tris–acetic acid buffer, pH 6·5. The volume of this solution was made up with the same buffer to one-quarter the volume of the original extract.

Second ammonium sulphate fractionation. The solution of ATPase from the previous step was further fractionated with $(NH_4)_2SO_4$. The fraction precipitated between 40 and 50% saturation with $(NH_4)_2SO_4$ was collected as in the preceding step. At this stage the enzyme was purified 60–80-fold and was used for some of the experimental work. If the enzyme at this stage was to be stored for experimental work it was dissolved in a 50% (v/v) glycerol solution in 10mM-tris–H_2SO_4 buffer, pH 7·5. If it was to be applied directly to a DEAE-cellulose column it was dissolved in 50ml. of 50mM-tris–acetic acid buffer (pH 6·5)/100ml. of original extract. If it was to be passed through a Sephadex-gel column it was dissolved in the same buffer but in 10ml./100ml. of extract.

Sephadex-gel column. The precipitate from the preceding step contained occluded $(NH_4)_2SO_4$, and unless it was dissolved in a large volume of buffer this prevented the enzyme adsorption of the enzyme on DEAE-cellulose. However, the enzyme is unstable in dilute solution and with larger preparations it was advantageous to dissolve the precipitate in a small volume of buffer and remove the $(NH_4)_2SO_4$ by passing the solution through a Sephadex-gel column. The column was equilibrated with the 50mM-tris–acetic acid buffer, pH 6·5, in which the enzyme was dissolved and the enzyme was eluted with the same buffer. In the preparation cited in Table 1 a column (2cm. diam. × 30cm. long) of Sephadex G-50 was used.

DEAE-cellulose fractionation. The ATPase is tightly bound to DEAE-cellulose and it is possible to adsorb the ATPase and allow much inactive protein to pass through. When a gradient elution was used the ATPase was obtained in dilute solution and with poor yields. Good yields were obtained if a flat pad (3cm. diam. × 1cm. high in the preparation cited in Table 1) of DEAE-cellulose was used instead of a long column. The enzyme was applied in 50mM-tris–acetic acid buffer, pH 6·5, the pad washed with 10ml. of the same buffer at 0·1M-concentration and the enzyme eluted with the same buffer at 0·25M. The ATPase was obtained in the minimum volume of buffer by watching for the more concentrated buffer and then collecting 1ml. fractions. In this way the ATPase is obtained in good yield (up to 90% of that applied to the column) with an overall purification of up to 130-fold and with specific activity up to 20·7 units/mg. of protein.

Storage of the enzyme. The enzyme is stored in 50% (v/v)

Table 1. *Purification of mitochondrial ATPase*

The data for a typical purification following the steps described in the text. The extract was the supernatant from 1·2g. of acetone-desiccated mitochondria suspended in 50ml. of water; 5ml. of 0·5M-tris–H_2SO_4 buffer, pH 7·5, was added before centrifugation.

Stage	Volume (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Specific activity (units/mg.)	Purification	Yield (%)
(1) Extract	50	0·66	4·4	0·15	1	100
(2) First $(NH_4)_2SO_4$ fraction	12·5	3·5	1·12	3·1	20	132
(3) Second $(NH_4)_2SO_4$ fraction	5·0	9·6	0·86	11·1	74	145
(4) Eluate from Sephadex-gel column	8·2	2·9	0·40	7·2	48	72
(5) Eluate from DEAE-cellulose	4·6	3·9	0·20	19·5	130	54

Table 2. *Decay of ATPase activity at 0° and 25°*

Extracted ATPase (unpurified, specific activity 0·16 unit/mg.) was incubated in 25mM-tris–HCl buffer, pH 7·4, under the indicated conditions. Activity was measured under the standard assay conditions and converted into units of ATPase activity by using the calibration curve shown in Fig. 1. The amounts of P_i released in 10min. when Mg^{2+} was omitted from and/or 2,4-dinitrophenol (0·5mM) added to the standard assay medium are expressed relative to the amount of P_i released under the standard assay conditions taken to be 1·00.

Treatment	% of initial activity	Relative amounts of P_i released in different assay media: Mg^{2+} omitted	Mg^{2+} omitted, dinitrophenol added	Dinitrophenol added
Fresh extract	100	0·08	0·16	1·50
3hr. at 0°	42	0·07	0·17	1·45
5hr. at 25°	103	0·06	0·13	1·41
3hr. at 25° followed by 3hr. at 0°	39	0·08	0·17	1·48

Table 3. *Effect of various compounds on the stability of partially purified ATPase*

ATPase purified by two $(NH_4)_2SO_4$ precipitations (specific activity 9·2units/mg.) was incubated in 10mM-tris–HCl buffer, pH 7·4, under the indicated conditions. Activity was measured under the standard assay conditions and converted into units of enzyme by using the calibration curve shown in Fig. 1.

Addition	% of initial activity after incubation: 30min. at 0°	2hr. at 25°	30min. at 45°
None	38	10	8
ATP (2·5mM), pH 7·5	46	130	97
ADP (2·5mM), pH 7·5	38	110	91
$MgSO_4$ (neutralized)	51	3	0
EDTA (2·5mM), pH 7·5	42	35	4
2,4-Dinitrophenol (1·0mM)	78	6	7
Acetone (10%, v/v)	49	8	0
Glycerol (50%, v/v)	127	110	95

glycerol in 10mM-tris–H_2SO_4 buffer, pH 7·5, at −20°. The activity declines to about one-quarter of the initial value in a month.

RESULTS

Starch-gel electrophoresis. Electrophoresis of the crude extract showed several bands of protein superimposed on a general background of protein, but staining for ATPase activity revealed only one band of activity. This band was intensified when 2,4-dinitrophenol (0·5mM) was incorporated into the medium, but no additional bands of ATPase activity appeared. Electrophoresis of the enzyme purified by two ammonium sulphate fractionations showed two bands of protein, but only the faster, more intense, band had ATPase activity, which was again enhanced by 0·5mM-2,4-dinitrophenol. It has not been possible to perform electrophoresis of the most highly purified preparations because of their instability in the absence of glycerol or ATP.

Stability. The effect of storing the crude extract of the ATPase at 25° and 0° is shown in Table 2. The activity is stable at 25°, but declines rapidly at 0°. The relative activities under the various conditions of assay remain virtually constant although the absolute activities fall to about half of the initial values. Preincubation at 25° did not prevent the inactivation at 0°, showing that the instability at 0° is not caused by some factor that is unstable at higher temperatures. Prolonged (48hr.) storage of the crude extract at 0° led to complete inactivation of the ATPase activity.

Table 3 shows the effect of various agents on the stability of the partially purified ATPase. Comparison of this Table with Table 2 shows that after purification the ATPase is much less stable at both 0° and 25°. The stabilizing effect of glycerol is note-

worthy, not only for its practical application to storage of the enzyme, but because it increases the stability of the enzyme over the whole range of temperatures tested, in contrast with ATP and ADP, which have little or no effect at 0°.

Substrate specificity. The following compounds were tested as substrates at three concentrations (0·1, 0·5 and 2·5 mM), each concentration at three pH values (pH 5·0, 50 mM-acetic acid–sodium hydroxide; pH 7·4 and pH 9·0, 50 mM-tris–hydrochloric acid), each test being made in the presence and absence of Mg^{2+} (when added the concentration of Mg^{2+} was equal to that of the compound under test). In all cases 0·8–1·0 unit of ATPase purified by two ammonium sulphate fractionations was used in 2 ml. of medium and incubation was for 10 min. at 25°. The compounds tested (all as sodium salts) were: inorganic pyrophosphate, inorganic tripolyphosphate, AMP, ribose 5-phosphate, phenyl phosphate and *p*-nitrophenyl phosphate. There was no detectable liberation of P_i in any test and the activity of the enzyme towards these compounds must be less than 0·1% of the activity towards ATP under the conditions of the standard assay.

With ADP as substrate (ADP concentration was 2·5 mM and the other conditions were as for the standard assay) the enzyme activity with 0·93 unit of enzyme purified by two ammonium sulphate fractionations was 2% of that with ATP as substrate. With 0·81 unit of enzyme at the final stage of purification the activity with ADP as substrate was only 0·9% of that with ATP. These results suggest that ADP is hydrolysed indirectly via the formation of ATP catalysed by traces of an adenylate kinase.

With ITP as substrate (2·5 mM-ITP replacing ATP in the standard assay) the enzyme activity was 87% of that with ATP as substrate.

Metal ion activation. The purified enzyme showed very little activity in the absence of an added bivalent metal cation and this feeble activity was further decreased in the presence of EDTA, which indicates that the enzyme has an absolute requirement for a metal ion activator (Table 4). The enzyme is not highly specific with regard to metal ion activators and a similar pattern of activation is observed with ATP or ITP.

The effect of 2,4-dinitrophenol depends very much on which metal ion activator is present when ATP is the substrate, but stimulation of activity by 2,4-dinitrophenol has not been observed with ITP as substrate.

Effect of 2,4-dinitrophenol. The effect of 2,4-dinitrophenol on the ATPase activity of partially purified enzyme at three pH values is shown in Fig. 2. Maximal stimulation is consistently observed at 0·5 mM-2,4-dinitrophenol; higher concentrations of 2,4-dinitrophenol inhibit the ATPase activity. No reproducible peaks of stimulation have been observed at lower concentrations of 2,4-dinitrophenol although individual experiments may show small peaks, e.g. those in the curve at pH 8·5 in Fig. 2.

At pH 7·5 and the same conditions as those given in Fig. 2, *p*-nitrophenol produces similar effects to those of 2,4-dinitrophenol. Maximal stimulation occurred at 0·2 mM-*p*-nitrophenol, but was only 21%.

Effect of pH. Fig. 3 shows the effect of pH on the ATPase activity of the crude extract in the presence of Mg^{2+}, 2,4-dinitrophenol and EDTA. The pH optimum is about pH 8·5 in the presence or absence of added Mg^{2+}, but is shifted towards pH 8·0 by the presence of 2,4-dinitrophenol. The increase in activity produced by 2,4-dinitrophenol is almost as great in the absence of added Mg^{2+} as when Mg^{2+} is added, and the effects of 2,4-dinitrophenol and Mg^{2+} appear to be additive, suggesting that two enzymes

Table 4. *Metal ion requirement of purified ATPase*

The conditions were: tris–HCl buffer, pH 7·4 (50 mM), 25°, 10 min. incubation, ATP (5 mM), ITP (3 mM), metal ion concentration (2·5 mM), 2,4-dinitrophenol (when present, 0·5 mM), EDTA (when present, 0·5 mM), volume 2·0 ml. In each test with ATP as substrate 6·2 μg. of enzyme (specific activity 17·3 units/mg. of protein) was used, except when no metal ions were added or in the presence of EDTA, when 310 μg. was used. In the experiments with ITP in the absence of 2,4-dinitrophenol 5·7 μg. of enzyme (specific activity 18·9 units/mg. of protein) was used in each test. The data for the effects of 2,4-dinitrophenol on the activity of the enzyme with ITP as substrate are taken from a series of experiments in which 0·08–0·12 unit of enzyme of specific activity not less than 14 units/mg. of protein was used in each test. P_i released is given as a percentage of that released in the presence of Mg^{2+} with ATP as substrate. In addition to the ions listed in the Table the following were tested but produced no activation of the enzyme, with ATP as substrate, in the presence or absence of 2,4-dinitrophenol: $BeSO_4$, $CuSO_4$, $HgCl_2$, $Pb(NO_3)_2$ (tris–HNO_3 buffer), $BaCl_2$, $Al_2(SO_4)_3$, $FeCl_3$, $CrCl_3$.

Substrate... ...	Relative activity			
Addition	ATP	ITP	ATP+ dinitrophenol	ITP+ dinitrophenol
None	0·2	—	0·35	—
EDTA	0·04	—	0·1	—
$MgSO_4$	100	100	130	44
$CoCl_2$	96	78	101	—
$ZnSO_4$	85	33	74	—
$FeSO_4$	58	50	73	—
$MnCl_2$	47	47	43	33
$CdSO_4$	28	16	25	—
$NiSO_4$	19	9	47	7
$CaCl_2$	7	2	8	—

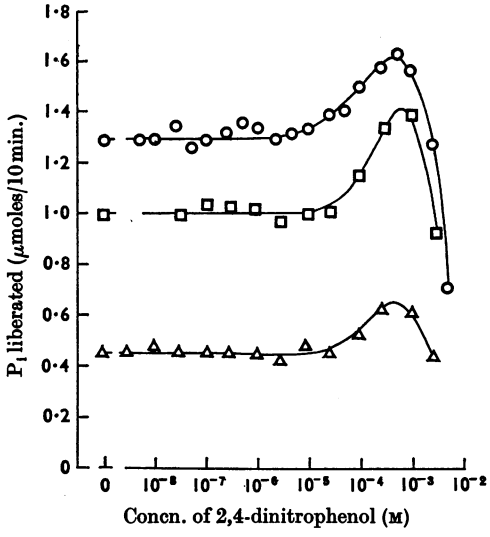

Fig. 2. Effect of 2,4-dinitrophenol on purified ATPase (specific activity 11 units/mg. of protein) at three pH values. The concentrations of ATP and Mg^{2+} were 2·5 mM and the temperature was 25° in all experiments. The volume was 2·0 ml. ○, Tris–HCl buffer, pH 8·5 (50 mM); □, tris–HCl buffer, pH 7·5 (50 mM); △, tris–acetic acid buffer, pH 6·5 (50 mM).

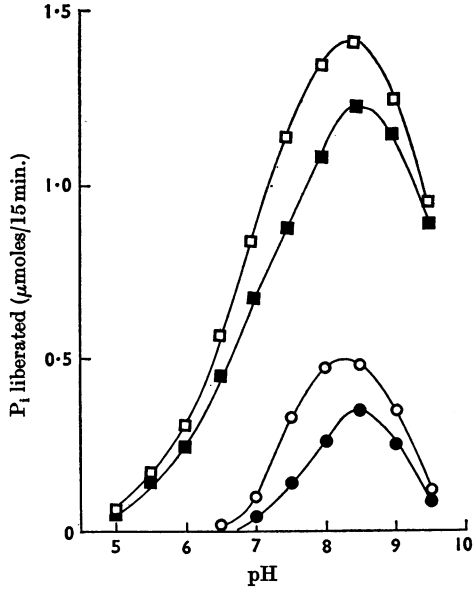

Fig. 3. Effect of pH on extracted but unpurified ATPase (specific activity 0·13 unit/mg. of protein) in the presence and absence of Mg^{2+} and 2,4-dinitrophenol. Buffer was 50 mM-tris adjusted to the indicated pH with acetic acid. All assay mixtures contained ATP (2·5 mM) and incubations were for 15 min. at 25°. The volume was 2·0 ml. □, $MgSO_4$ (2·5 mM) and 2,4-dinitrophenol (0·5 mM) added; ■, $MgSO_4$ (25 mM) added; ○, 2,4-dinitrophenol (0·5 mM) added; ●, no addition.

were present. However, when 1 mM-EDTA was present there was no detectable activity in the presence or absence of 2,4-dinitrophenol over the whole range of pH tested, which indicates that activity in the absence of any added Mg^{2+} is due to metal ions present in the crude extract.

The effect of pH on the activity of the purified enzyme (2·5 mM-ATP, 2·5 mM-magnesium sulphate, buffers as in Fig. 3) is very similar to its effect on the Mg^{2+}-activated crude enzyme.

Kinetics. The great variation in the values reported for the stability constant of the ATP–Mg^{2+} complex (reviewed by Bock, 1960) and the large activity coefficients for highly charged ions such as ATP^{4-} render calculations of the concentrations of the ATP–Mg^{2+} complex, free ATP and free Mg^{2+} extremely unreliable. However, the stability constant is sufficiently large that a useful approximation can be made by assuming that it is infinite, i.e. the concentration of free ATP or Mg^{2+}, whichever is present in excess, is the difference between the total concentrations of ATP and Mg^{2+}, and the concentration of the ATP–Mg^{2+} complex is equal to the total concentration of the component present in lower concentration. With this approximation, if [X] is the total concentration of the component present in greater concentration and [Y] the total concentration of the other component, and if X is a competitive inhibitor (inhibitor constant K_i) and K_m applies to the complex XY, then the rate equation (personal communication from Dr K. Dalziel) is:

$$v = \frac{V}{\left(1-\frac{K_m}{K_i}\right)+\frac{K_m}{[Y]}\left(1+\frac{[X]}{K_i}\right)}$$

At a fixed concentration of X the plot of $1/v$ against $1/[Y]$ is linear, but if X is a non-competitive inhibitor the plot is not linear. The intercept on the $1/v$ axis is $(1-K_m/K_i)/V$, i.e. the apparent maximum velocity is greater than when the [X]/[Y] ratio is 1:1.

In the absence of added 2,4-dinitrophenol the rate was higher when the [ATP]/[Mg^{2+}] ratio was 1:1, i.e. when the concentrations of free ATP and Mg^{2+} were minimal, than when an excess of either ATP or Mg^{2+} was present (Fig. 4). The linearity of the curves when either ATP or Mg^{2+} was present at a fixed total concentration shows that the inhibition by free ATP or Mg^{2+} is competitive. These findings suggest that the enzyme–ATP–Mg^{2+} complex is formed by combination of the enzyme with the ATP–Mg^{2+} complex and not, or more slowly, by

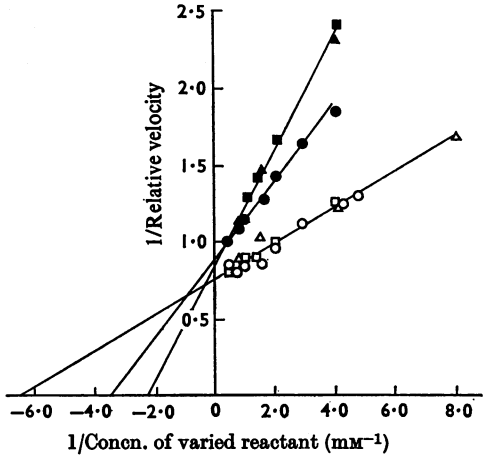

Fig. 4. Effect of ATP and Mg^{2+} concentrations. All measurements were made in 50mM-tris–HCl buffer, pH 7·4, at 25° and rates are expressed relative to those under the standard assay conditions as described in the text. ● and ○, Effect of varying the concentrations of both ATP and Mg^{2+}, both being present in equal concentration: 16μg. of enzyme (specific activity 16units/mg. of protein) was added/10ml. of medium. ▲ and △, Effect of varying the concentration of Mg^{2+} with 2·5mM-ATP: 10μg. of enzyme (specific activity 15 units/mg. of protein) was added/10ml. of medium. ■ and □, Effect of varying the concentration of ATP, with 2·5mM-Mg^{2+}: 13μg. of enzyme (specific activity 20units/mg. of protein) was added/10ml. of medium. Determinations were made in the presence (○, △ and □) and absence (●, ▲ and ■) of 0·5mM-2,4-dinitrophenol.

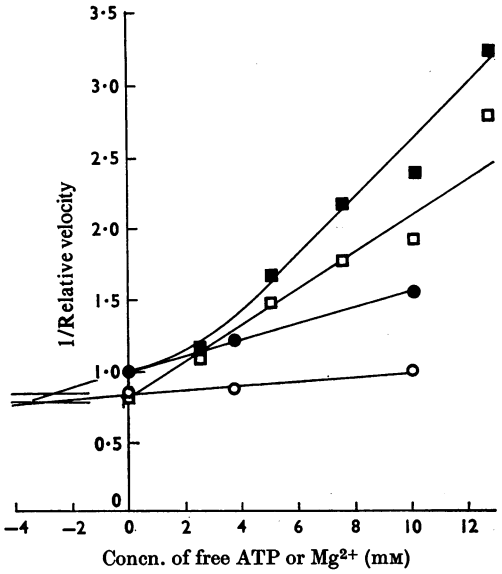

Fig. 5. Inhibition by high concentrations of free ATP and Mg^{2+}. ■ and □, Effect of varying the concentration of ATP, with 2·5mM-Mg^{2+}. ● and ○, Effect of varying the concentration of Mg^{2+}, with 2·5mM-ATP. A 14·5μg. portion of enzyme (specific activity 17units/mg. of protein) was added/10ml. of medium. Determinations were made in the presence (□ and ○) and absence (■ and ●) of 0·5mM-2,4-dinitrophenol.

reaction of an enzyme–ATP complex with Mg^{2+} or reaction of an enzyme–Mg^{2+} complex with ATP.

Fig. 4 shows that the major effect of 2,4-dinitrophenol is to lower the K_m for the ATP–Mg^{2+} complex from 0·29mM to 0·15mM, but it also produces a small (20%) increase in V. In steady-state systems (as opposed to systems where the intermediates are in equilibrium with the free enzyme and substrate) a modifier that produces a change in V will also alter the K_m, but in the present case the change in V is not only too small but is in the wrong direction to account for the change in K_m. Thus 2,4-dinitrophenol acts on this enzyme by affecting the binding of the substrate directly and not by affecting the rate of dissociation of the products as may be the case with myosin ATPase (Blum & Felauer, 1959).

The inhibitory effects of higher concentrations of free ATP and Mg^{2+} are shown in Fig. 5. The inhibition produced by Mg^{2+} is simple (linear or first-order) and, although the intercepts are rather acute, the K_i can be estimated from the point at which the extrapolated curve has a value equal to V. The K_i is approx. 3mM in the presence or absence of 2,4-dinitrophenol. However, the inhibition

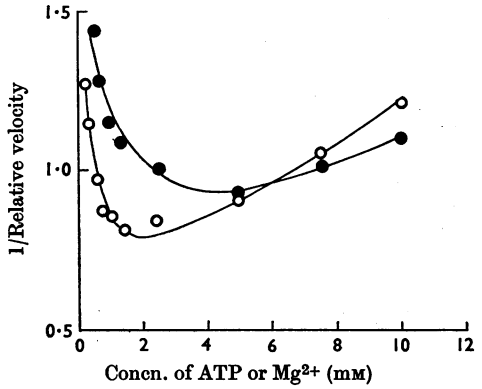

Fig. 6. Inhibition by high concentrations of the ATP–Mg^{2+} complex. ATP and $MgSO_4$ were present in equal concentrations. A 13μg. portion of enzyme (specific activity 20units/mg. of protein) was added/10ml. of incubation medium. Determinations were made in the presence (○) and absence (●) of 0·5mM-2,4-dinitrophenol.

produced by a particular concentration of Mg^{2+} is decreased by 2,4-dinitrophenol because, as shown by the results given above, 2,4-dinitrophenol lowers

the K_m for the ATP–Mg^{2+} complex. The inhibition produced by high concentrations of free ATP is complex (second-order or parabolic) and is markedly affected by 2,4-dinitrophenol.

When ATP and Mg^{2+} are present in high but equal concentrations, i.e. there is a high concentration of the ATP–Mg^{2+} complex with minimal concentrations of free ATP and Mg^{2+}, the rate of reaction is diminished (Fig. 6). The K_i for this inhibition can be estimated from the concentration at which the maximal rate of reaction occurred and the previously determined value for the K_m (see Dixon & Webb, 1964). In the presence of 0·5mM-2,4-dinitrophenol the K_i is decreased from 62mM to 26mM.

DISCUSSION

Comparison with other preparations of mitochondrial ATPase. The present preparation has several properties in common with the ATPase coupling factor described by Pullman *et al.* (1960), e.g. substrate specificity, metal ion activator requirements, the effects of 2,4-dinitrophenol, the rapid decline in rate as the reaction proceeds (Selwyn, 1965), indicating a low K_i for one or more of the products, the unusual property of cold-lability and the insensitivity of the extracted ATPase to inhibition by oligomycin (Selwyn & Chappell, 1962). Only minor quantitative differences between the two preparations have been observed, e.g. in the rates of inactivation at low temperatures. The increase in total activity during purification, which is associated with a heat treatment in the procedure of Pullman *et al.* (1960), is associated with an ammonium sulphate fractionation in the present method and is probably caused by removal of an inhibitory factor rather than by modification of the tertiary structure of the protein. These similarities suggest that the two preparations, which start from the same source material, are fundamentally the same protein, though there may be minor differences in any modification of the native state of the protein that may occur in the extraction and purification procedures.

During the development of the purification procedure all fractions were examined for ATPase activity, but no biphasic distribution of activity was found. Although about 25% of the ATPase activity remains in the insoluble residue after one extraction of the acetone-dried ox-heart mitochondria, repeated extraction of the residue yields further, but diminishing, amounts of the same soluble ATPase (the instability of the enzyme and the time-scale of the process make repeated extraction unprofitable for preparative purposes). This, together with the results of starch-gel electrophoresis, the parallel disappearance of Mg^{2+}-activated and 2,4-dinitrophenol-stimulated ATPase activity during cold inactivation and the finding that all the ATPase activity is cold-labile, suggests very strongly that there is only one ATPase in the extracts of acetone-dried ox-heart mitochondria. Pullman *et al.* (1960) have also found only one ATPase in extracts of ox-heart mitochondria.

However, Beyer (1960) observed two apparently soluble ATPases; one of these was Mg^{2+}-dependent and 2,4-dinitrophenol-stimulated and seems to correspond to the one described here, and the other, released on prolonged ultrasonic treatment, required no metal ion activator and was not stimulated by 2,4-dinitrophenol. Since some of the ATPase is tightly bound to the mitochondria (M. E. Pullman, unpublished work, quoted by Racker, 1961) and certain submitochondrial particles contain bound Mg^{2+} (Gamble & Lehninger, 1956), it is possible that the second ATPase was really bound to very small particles containing bound Mg^{2+}.

Penniall (1960) has reported that crude extracts of acetone-dried rat-liver mitochondria show multiple peaks of stimulation of ATPase by 2,4-dinitrophenol and has interpreted this as evidence for the presence of several ATPases in these extracts. Attempts to repeat Penniall's (1960) observations have been equivocal; only small peaks, such as those in Fig. 2, have been found with crude or partially purified extracts of acetone-dried rat-liver and ox-heart mitochondria. These peaks although usually present are not constant in position and Penniall's (1960) data also show that the position of the peaks varied in different preparations. An alternative explanation for Penniall's (1960) observations is that there is one ATPase bound to several different modifiers that are either displaced by 2,4-dinitrophenol at different concentrations or alter the apparent affinity of the enzyme for 2,4-dinitrophenol, and his data do not justify his claim that they are direct evidence for the presence of several ATPases. This explanation can also account for the observations of Myers & Slater (1958), Wadkins (1961) and Hemker (1963) on the ATPase of whole mitochondria or submitochondrial particles. In these cases there is the added complication of the effects of pH on the solubility of phenolic uncoupling agents in the lipid phases of mitochondria or submitochondrial particles (Hemker & Hulsmann, 1961). Further, the finding that the effect of 2,4-dinitrophenol on the soluble ATPase is produced mainly by a lowering of the K_m for the ATP–Mg^{2+} complex, which is in accord with Cooper's (1958*a*,*b*) observations on the ATPase of mitochondrial fragments produced by digitonin treatment, indicates the need for considering the effects of pH or 2,4-dinitrophenol on the availability of Mg^{2+}. This finding also means that any deductions drawn from the apparently additive nature of Mg^{2+} activation

and 2,4-dinitrophenol stimulation must be viewed with caution unless a wide range of Mg^{2+} concentrations has been used and the possibility of traces of Mg^{2+} in the enzyme preparation eliminated.

The properties of the ATPase described in the present paper do not distinguish it from the actomyosin-like ATPase described by Ohnishi & Ohnishi (1962) and Neifakh & Kazakova (1963). In attempts to prepare the actomyosin-like ATPase from rat-, rabbit- and chicken-liver mitochondria by extraction with 0·6M-potassium chloride, only weak ATPase activity was obtained and it was not possible to observe the actomyosin characteristics of super-precipitation and viscosity changes on addition of ATP, as a preliminary to comparing the two ATPases. Conover & Bárány (1966), in an extensive investigation, also failed to find satisfactory evidence for the presence of a myosin-like protein in extracts of liver mitochondria.

Thus there is, at present, no conclusive evidence for the presence in any mitochondrial extracts of more than one protein having ATPase activity or for any type of soluble ATPase from mitochondria other than a Mg^{2+}-dependent 2,4-dinitrophenol-stimulated enzyme.

ATPase in mitochondria. This failure to find satisfactory evidence for more than one type of ATPase in extracts of mitochondria and the great similarity between the present preparation and the other highly purified ATPase from mitochondria (Pullman *et al.* 1960) suggests that most of the ATPase activity of intact mitochondria may be due to a single protein. However, the ATPase activity of whole mitochondria has properties that are markedly different from those of the soluble purified ATPases. On the basis of indirect evidence from the properties of the ATPase activities of different submitochondrial particles Selwyn & Chappell (1962) postulated that in intact mitochondria the ATPase is combined with at least two factors that modify its activity.

Racker and his colleagues have isolated several factors that, alone or together, restore to the purified ATPase properties that are possessed by the ATPase of whole mitochondria, e.g. certain factors inhibit the ATPase and enhance the stimulation by 2,4-dinitrophenol (Zalkin & Racker, 1965) and a particulate fraction from mitochondria restores the sensitivity of the ATPase to inhibition by oligomycin (Kagawa & Racker, 1966*a*,*b*).

The kinetic data described in the present paper support the view that on extraction the ATPase is altered by removal of accessory factors rather than by a fundamental alteration in the protein molecule, since the ATPase activities of submitochondrial particles show similarities to the soluble ATPase. Cooper's (1958*a*,*b*) observations on the effects of 2,4-dinitrophenol on the ATPase of submitochondrial particles produced by digitonin treatment have been mentioned already. The effects of varying the $[ATP]/[Mg^{2+}]$ ratio reported in the present work are similar to the observations of Kielley & Kielley (1953) on mechanically disrupted mitochondria and of Ulrich (1964) on particles prepared by deoxycholate treatment of mitochondria: these workers also concluded that the ATP–Mg^{2+} complex is the important substrate.

Mode of action of 2,4-dinitrophenol. The finding that the effect of 2,4-dinitrophenol on the soluble ATPase is mainly on the K_m for the ATP–Mg^{2+} complex, which is in marked contrast with its effect on myosin ATPase, is significant because the apparent similarity between the effects of 2,4-dinitrophenol on these two ATPases is the basis for a suggestion (Boyer, 1965) about the mode of action of phenols as uncoupling agents and about the nature of the non-phosphorylated high-energy intermediate. Boyer (1965) postulates that the intermediate is an acyl thio ester and that 2,4-dinitrophenol uncouples by forming an unstable acyl phenyl ester. If the action of 2,4-dinitrophenol on the soluble ATPase is related to its action as an uncoupling agent then this double-displacement reaction should produce an increase in V and either no alteration or an increase in K_m.

Other double-displacement reactions such as the formation of a phenyl ester with the β-phosphate residue of ADP or a phenyl phosphate ester are also inconsistent with the kinetic evidence. The latter possibility, formation of a phenyl phosphate ester, is not consistent with the indirect evidence obtained from studies on whole mitochondria (reviewed by Sanadi, 1965) and is also at variance with the present findings that *p*-nitrophenyl phosphate is not a substrate for the soluble ATPase, although *p*-nitrophenol produces effects similar to those of 2,4-dinitrophenol on the soluble ATPase and on whole mitochondria (Parker, 1958). Boyer (1958) found that 2,4-dinitrophenyl phosphate was stable in neutral aqueous solution, but this did not eliminate phenyl phosphates as intermediates as they might be hydrolysed rapidly by an enzymic reaction.

Mitchell's (1961, 1966) hypothesis of chemiosmotic coupling involves an ATPase as the central feature of the coupling mechanism. However, a fundamental feature of this hypothesis is that phenolic uncoupling agents act by facilitating the transfer of protons across the mitochondrial membrane and this does not account for the effects of 2,4-dinitrophenol on the isolated ATPase. It is possible that these effects on the ATPase are not related to the action of 2,4-dinitrophenol as an uncoupling agent, but if they are then the chemiosmotic hypothesis requires, at the least, considerable modification.

The present findings are in accord with the views

of Selwyn & Chappell (1962), who postulated that phenolic uncoupling agents act by displacing an inhibitory factor from the ATPase in whole mitochondria. The binding site for phenols would still be present on the isolated enzyme, but only certain phenols produce stimulation of the ATPase activity.

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