BBA 45618

THE ROLE OF THE ADP–ATP EXCHANGE ENZYME IN OXIDATIVE PHOSPHORYLATION

G. S. P. GROOT AND S. G. VAN DEN BERGH

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)

(Received May 31st, 1967)

SUMMARY

1. A nucleoside diphosphate kinase (EC 2.7.4.6) has been isolated from liver mitochondria that is rather specific for ATP as phosphate donor. It catalyses an ADP–ATP exchange reaction.

2. The isolated enzyme is inhibited by ADP in concentrations higher than 1 mM. The reaction is stimulated by both ferri- and ferrocytochrome *c*, but the inhibition by high ADP concentrations is not relieved.

3. The isolated enzyme is insensitive to 2,4-dinitrophenol and oligomycin. It appears to become sensitive to 2,4-dinitrophenol but not to oligomycin on addition of freshly prepared digitonin particles. The inhibition is due to the formation of ADP, catalysed by the adenosine triphosphatase in the digitonin particles.

4. The oligomycin-sensitive ADP–ATP exchange activity in the mitochondria is not affected by extraction of the nucleoside diphosphate kinase.

5. The isolated enzyme has no effect either on the P/O ratio or on the ATP-driven nicotinamide nucleotide transhydrogenase of submitochondrial particles.

6. A role of this enzyme in oxidative phosphorylation seems improbable.

INTRODUCTION

The ADP–ATP exchange reaction has been extensively studied in intact mitochondria[1–5], digitonin particles[6–10] and sonic particles[11]. In intact mitochondria this reaction is inhibited under carefully defined conditions[4] by uncouplers and inhibitors of oxidative phosphorylation. It is, therefore, widely believed that the ADP–ATP exchange reaction represents the last step in the phosphorylation mechanism,

$$X \sim P + ADP \rightleftharpoons X + ATP$$

WADKINS AND LEHNINGER[6,8] have isolated from liver mitochondria and from digitonin particles an easily extracted enzyme that catalyses an ADP–ATP exchange reaction. The isolated enzyme is completely insensitive to uncouplers or inhibitors of oxidative phosphorylation[6], but can be rendered sensitive to dinitrophenol on incubation with digitonin particles[12]. WADKINS AND LEHNINGER[8] were also able to show

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl-2)-benzene.

that mitochondria from which the enzyme was extracted could reincorporate the enzyme with a concomitant rise in the P/O ratio with β-hydroxybutyrate, succinate or ascorbate as substrate. They concluded that the enzyme acts as a coupling factor, especially at the third site of phosphorylation.

More direct evidence for a role of the enzyme in oxidative phosphorylation came from the observation that the inhibition of the exchange reaction by high concentrations of ADP could be specifically relieved by addition of ferrocytochrome *c*. The effect of ferrocytochrome *c* was abolished by oligomycin and potentiated by 2,4-dinitrophenol[13,14].

RACKER and co-workers[15] have isolated from beef-heart mitochondria a coupling factor (F_1) that is required in submitochondrial particles for respiratory-chain phosphorylation and for all reactions associated with oxidative phosphorylation that involve a transphosphorylation step with ATP. F_1 also catalyses the hydrolysis of ATP and other nucleoside triphosphates, and this reaction is inhibited by ADP. However, F_1 does not catalyse an ADP–ATP exchange[16,17]. Moreover, ZALKIN, PULLMAN AND RACKER[17] found no dinitrophenol-sensitive ADP–ATP exchange activity in submitochondrial particles from beef heart that catalysed an ATP–P_i exchange. They concluded that the exchange observed in submitochondrial particles is mainly due to adenylate kinase and other heat-stable enzymes that form [^{14}C]ATP from [^{14}C]ADP.

The purpose of this paper is to examine the relationship between the enzyme described by WADKINS and co-workers and the ADP–ATP exchange activity in intact mitochondria and digitonin particles.

METHODS AND MATERIALS

Mitochondria were prepared from beef liver following essentially the procedure for beef heart described by CRANE, GLENN AND GREEN[18], using 0.25 M sucrose–1 mM EDTA as isolation medium. The mitochondrial pellet was suspended in the isolation medium (to a concentration of about 30 mg protein per ml) and stored overnight at 0° with continuous stirring. The isolation of the enzyme was carried out at 0°. The suspension was centrifuged for 30 min at 12000 × *g*, and the cloudy supernatant dialysed several times against 1 mM phosphate buffer (pH 7.4). After adjustment of the pH to 7.4, inactive protein was removed by centrifugation at 12000 × *g*. From the supernatant the protein precipitating between 25 g $(NH_4)_2SO_4$ and 65 g $(NH_4)_2SO_4$ per 100 ml was collected and dissolved in 1 mM phosphate buffer (pH 7.4). This solution was brought on a DEAE-cellulose column previously equilibrated with 1 mM phosphate buffer (pH 7.4). The protein (11.3 g) eluted with 50 mM phosphate buffer (pH 7.4) had a specific activity of 1.6 μmoles/min per mg and was concentrated by repeating the precipitation procedure with $(NH_4)_2SO_4$. The precipitate was dissolved in 1 mM phosphate buffer (pH 6.0) and dialysed several times against the same buffer. After centrifugation the solution was brought on a CM-cellulose column equilibrated with 1 mM phosphate buffer (pH 6.0). The protein was eluted with a logarithmic gradient[19] from 1 to 50 mM phosphate buffer (pH 6.0). The active fractions, containing 3.9 g protein, were pooled. They had a specific activity of 3.2 μmoles/min per mg and were concentrated by repeating the $(NH_4)_2SO_4$ precipitation procedure. The precipitate was dissolved in 50 mM phosphate buffer (pH 7.4) and dialysed several times against the same buffer. The solution was then brought on a hydroxyapatite column,

equilibrated with 50 mM phosphate buffer (pH 7.4), and the protein was eluted with a steep, linear gradient[19] from 50 to 500 mM phosphate buffer (pH 7.4). The active fraction contained 20 mg of protein with a specific activity of 44.4 μmoles/min per mg.

The enzyme was also prepared from rat-liver mitochondria, prepared according to MYERS AND SLATER[20], usually omitting the chromatography on CM-cellulose.

Digitonin particles from rat-liver mitochondria were prepared according to DEVLIN AND LEHNINGER[21]. Mitochondria from beef heart were prepared as described by CRANE, GLENN AND GREEN[18] and submitochondrial particles ('A' particles) according to FESSENDEN AND RACKER[22]. Protein was determined according to the biuret method of GORNALL, BARDAWILL AND DAVID[23], as described by CLELAND AND SLATER[24]. Oxygen uptake was measured in differential manometers and phosphorylation was measured as described by SLATER[25].

The ADP–ATP exchange activity of the isolated enzyme was measured in a medium containing 30 mM Tris–HCl buffer (pH 7.4), 5.5 mM $MgCl_2$, 0.5 mM EDTA, and [8-^{14}C]ADP (100000–130000 disintegrations/min) and ATP as indicated in a final volume of 1 ml. In the recombination experiments the reaction medium contained 30 mM Tris–HCl buffer (pH 7.4), 1.5 mM $MgCl_2$, 0.5 mM EDTA and 25 mM sucrose. Mitochondrial ADP–ATP exchange activity was measured as described by GUILLORY AND SLATER[4]. The nucleotides were separated on a Dowex 1 formate column. ADP was eluted with 4 M HCOOH and ATP with 0.25 M HCl. The recovery of the nucleotides from the column was 95–100 % of that applied. The concentration of the nucleotides in the eluates was determined spectrophotometrically at 260 mμ using a molar extinction coefficient of $15.2 \cdot 10^6$ (ref. 4). Samples were dried under an infrared lamp in glass counting vials and dissolved in 0.2 ml methanol, 0.1 ml 1 M hyamine in methanol and 9.7 ml toluene, containing 4 g PPO and 50 mg POPOP per l. The vials were counted to a constant rate in a Nuclear Chicago Mark 1 liquid scintillation counter. The initial rate of the exchange reaction was calculated from the formula given by DUFFIELD AND CALVIN[26].

Nucleoside diphosphate kinase was measured in a medium containing 25 mM Tris–HCl buffer (pH 7.4), 2 mM $MgCl_2$, 0.5 mM EDTA, 30 mM glucose, 2 mM $NADP^+$, ADP and nucleoside triphosphate as indicated, 20 μg hexokinase (EC 2.7.1.1), and 10 μg glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The increase of the absorption at 340 mμ was followed in a Zeiss PMQII spectrophotometer. Corrections were made for the reaction of the nucleoside triphosphate with hexokinase by omitting ADP from the reaction mixture, and for adenylate kinase by omitting the nucleoside triphosphate.

The ATPase activity was measured as described by MYERS AND SLATER[20].

[8-^{14}C]ADP was purchased from Calbiochem and purified before using on a Dowex 1 formate column, as described by GUILLORY AND SLATER[4]. Nucleotides were obtained from Sigma Chemical Company and Boehringer und Söhne. DEAE-cellulose was obtained from Serva Entwicklungslabor, CM-cellulose from Sigma Chemical Company and hydroxyapatite from Calbiochem. Oligomycin was kindly provided by the Upjohn Chemical Company, aurovertin by Dr. H. A. LARDY and atractyloside by Dr. V. SPRIO. Purified F_1 was a gift from Dr. R. H. VALLEJOS. Cytochrome *c* was prepared from horse heart as described by KEILIN AND HARTREE[27] and purified according to MARGOLIASH[28]. Hexokinase and glucose-6-phosphate dehydrogenase were

obtained from Boehringer und Söhne. All other chemicals used were from the British Drug Houses Ltd.

RESULTS

The ADP–ATP exchange enzyme, isolated from calf-liver mitochondria, is readily purified to a high specific activity. The enzyme shows a typical protein absorption spectrum with a maximum at 279 mμ and a minimum at 250 mμ. The purified preparation contains neither adenylate kinase nor ATPase. From Table I it appears that the enzyme is relatively specific towards ATP as phosphate donor. GTP and UTP can also serve, but only with about 10 % and 6 % of the velocity of ATP, whereas CTP is inactive. In agreement with WADKINS AND LEHNINGER[6,7] the purified enzyme was found to be insensitive to 0.1 mM dinitrophenol or 2.5 μg oligomycin per μg enzyme. It was also insensitive to 0.5 μg aurovertin per μg enzyme, 0.5 μg atractyloside per μg enzyme or 0.1 mM avidin. *p*-Chloromercuribenzoate (0.1 mM) inhibited by 50 %.

TABLE I

SPECIFICITY OF THE ADP–ATP EXCHANGE ENZYME FOR PHOSPHATE DONOR

The velocity of the ADP–ATP exchange, nucleoside diphosphate kinase and adenylate kinase were measured as described in METHODS. The concentration of ADP was 0.9 mM and of the nucleoside triphosphate 2.8 mM; 30 μg purified enzyme was used. Except for the measurement of the exchange between ADP and ATP, a reaction temperature of 22° was used. The latter exchange was carried out at 0° with 6 μg purified enzyme and corrected to 22° by applying a factor (4.4) determined in separate experiments.

Phosphate donor	*Rate (μmoles/min per mg)*
ATP*	44.4
GTP	4.2
UTP	2.8
CTP	0.5
ADP**	0.09

* ADP–ATP exchange.
** Adenylate kinase.

In agreement with GLAZE AND WADKINS[13] the exchange enzyme was inhibited by ADP in concentrations higher than 1 mM. Addition of cytochrome *c* in the presence of ascorbate caused a slight stimulation of the exchange reaction over the whole range of ADP concentrations tested (see Fig. 1). However, in contrast to GLAZE AND WADKINS[13], the inhibition by ADP was not relieved by ferrocytochrome *c*, and a similar stimulation was found with cytochrome *c plus* ferricyanide, showing that the effect is not specific for the reduced cytochrome. Similar results were obtained with GTP as phosphate donor (Fig. 2). The stimulatory effect of cytochrome *c* remained the same between 10 and 100 μM. Also in contrast to the results of GLAZE AND WADKINS[13], 2,4-dinitrophenol and oligomycin were found to have no effect on the stimulation of the exchange reaction by cytochrome *c* (Table II).

In the absence of added Mg^{2+}, the ADP–ATP exchange reaction in freshly prepared digitonin particles from rat-liver mitochondria is sensitive to 2,4-dinitro-

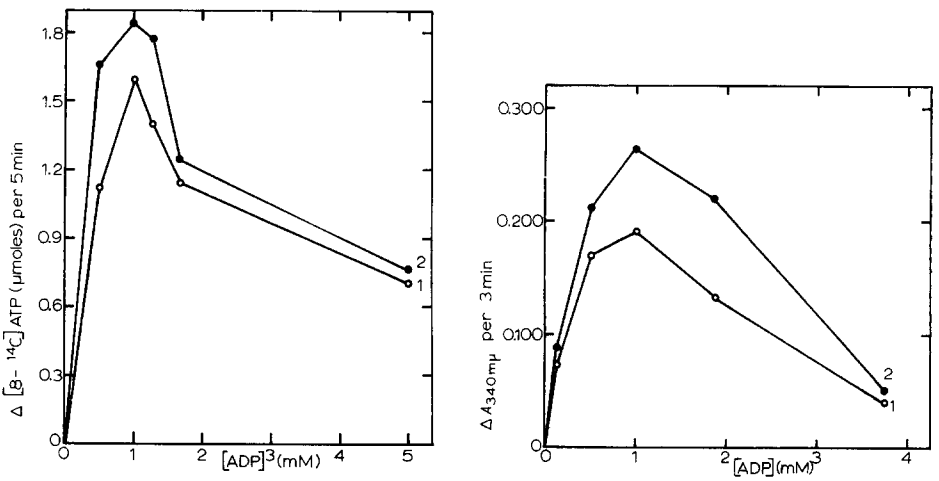


Fig. 1. Effect of ferrocytochrome *c* at different ADP concentrations on the activity of the soluble ADP–ATP exchange enzyme. The reaction medium described in METHODS contained 4.5 mM ATP, ADP as indicated (25600 counts/min), 10 mM ascorbate and 16 μg purified enzyme. Reaction time, 5 min. Temp., 10°. Curve 1, control; Curve 2, *plus* 10 μM cytochrome *c*.

Fig. 2. Effect of ferrocytochrome *c* at different ADP concentrations on the nucleoside diphosphate kinase reaction. The reaction medium described in METHODS contained 3 mM GTP, ADP as indicated, 8 mM ascorbate and 40 μg purified enzyme. Temp., 22°. Curve 1, control; Curve 2, *plus* 10 μM cytochrome *c*.

TABLE II

THE EFFECT OF 2,4-DINITROPHENOL AND OLIGOMYCIN ON THE STIMULATION OF THE SOLUBLE ADP–ATP EXCHANGE ENZYME BY CYTOCHROME *c*

1.1 mM [8-^{14}C]ADP, 8.9 mM ATP, 4 mM ascorbate and 3 μg purified enzyme were included in the reaction mixture described under METHODS. Temp., 25°. Reaction time, 7 min.

Additions	*Δ[8-^{14}C]ATP (μmoles)*
None	0.99
Cytochrome *c* (40 μM)	1.16
Cytochrome *c* (40 μM) + 2,4-dinitrophenol (0.1 mM)	1.12
Cytochrome *c* (40 μM) + oligomycin (5 μg)	1.14

phenol and oligomycin (Fig. 3). The total ADP–ATP exchange activity is greatly increased by the addition of Mg^{2+}, but the oligomycin-sensitive activity remains constant. The 2,4-dinitrophenol-sensitive activity, however, increases with increasing Mg^{2+} concentration. When the soluble exchange enzyme (either in the crude extract or the purified enzyme) is added to digitonin particles the activity of the soluble enzyme becomes partly inhibited by dinitrophenol (Fig. 4B), but not by oligomycin (Fig. 4A).

Since dinitrophenol, but not oligomycin, induces an appreciable ATPase activity in digitonin particles, the effect of ATPase on the soluble enzyme was studied by adding F_1, the ATPase isolated from beef-heart mitochondria. It has been shown that F_1 does not catalyse an ADP–ATP exchange reaction[17]. As is seen in Fig. 5, the

ADP–ATP exchange reaction was inhibited by the addition of F_1 and the inhibition was greater between 7 and 15 min (when the ADP concentration was between 1.9 and 2.3 mM) than between 0 and 15 min (when the ADP concentration increased from 1.2

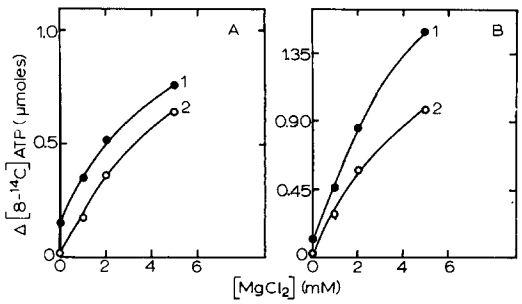
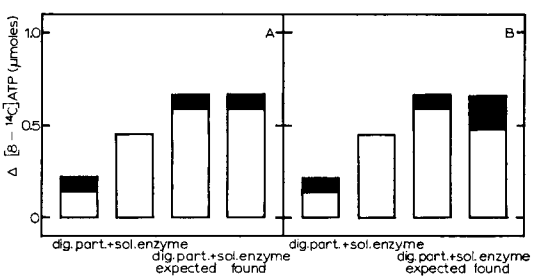


Fig. 3. Effect of added Mg^{2+} on the inhibition of the ADP–ATP exchange reaction by oligomycin and 2,4-dinitrophenol in digitonin particles. The reaction medium was as described in METHODS, except for $MgCl_2$ which was as indicated. Expt. A: 1.2 mg digitonin particles; Curve 1, control; Curve 2, *plus* 5 μg oligomycin. Expt. B: 1.9 mg digitonin particles; Curve 1, control; Curve 2, *plus* 0.5 mM 2,4-dinitrophenol. Reaction time, 5 min. Temp., 25°.



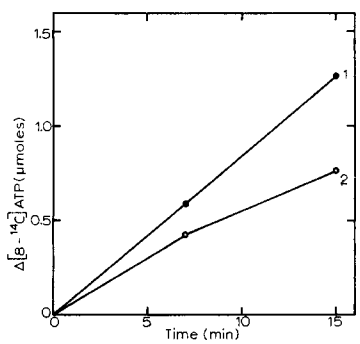


Fig. 4. Sensitivity to oligomycin and to 2,4-dinitrophenol of ADP–ATP exchange activity of digitonin particles, purified exchange enzyme and of a combination of the two. The reaction medium was as described in METHODS, with 7.0 mM ATP, 2.1 mM [8-^{14}C]ADP, 120 μg digitonin particles, 50 μg ADP–ATP exchange enzyme, 0.5 mM 2,4-dinitrophenol and 5 μg oligomycin. A: The shaded area represents the fraction of the ADP–ATP exchange activity that is sensitive to oligomycin. B: The shaded area represents the fraction of the ADP–ATP exchange activity that is sensitive to 2,4-dinitrophenol.

Fig. 5. Effect of ATPase (F_1) on the ADP–ATP exchange activity of the soluble enzyme. The reaction medium as described in METHODS contained 7.3 mM ATP, 1.2 mM [8-^{14}C]ADP and 20 μg purified enzyme. Curve 1, control. Curve 2, *plus* 170 μg F_1.

to 1.9 mM). Similar results were obtained when glucose and hexokinase were used as an ATP-splitting system. Increasing amounts of hexokinase brought about an increasing inhibition of the ADP–ATP exchange reaction (Table III).

When rat-liver mitochondria are incubated for a short time in 0.3 M $(NH_4)_2SO_4$ at 0–2°, much ADP–ATP exchange activity, insensitive to 2,4-dinitrophenol or oligomycin, is solubilized[8]. However, the oligomycin-sensitive ADP–ATP exchange remains completely in the mitochondria (Table IV). This is consistent with the finding of KEMP (unpublished) that extraction of rat-liver mitochondria with $(NH_4)_2SO_4$ had

TABLE III

EFFECT OF GLUCOSE *plus* HEXOKINASE ON THE SOLUBLE ADP–ATP EXCHANGE REACTION

7.0 mM ATP, 1.1 mM [8-^{14}C]ADP, 2.5 μg purified enzyme and 30 mM glucose were included in the reaction medium described in METHODS. Temp., 24°. Reaction time, 10 min.

Hexokinase (μg)	*Δ[8-^{14}C]ATP (μmole)*
0	0.96
3	0.66
6	0.47
12	0.35

TABLE IV

EFFECT OF $(NH_4)_2SO_4$ EXTRACTION ON THE OLIGOMYCIN-SENSITIVE ADP–ATP EXCHANGE REACTION IN RAT-LIVER MITOCHONDRIA

Reaction medium: 25 mM Tris–HCl (pH 7.4), 100 mM sucrose, 0.5 mM EDTA, 10 μM cytochrome *c*, 8.1 mM ATP, 1.1 mM [8-^{14}C]ADP in a final volume of 1 ml. A part of the mitochondrial preparation was extracted for 10 min at 0° with 0.3 M $(NH_4)_2SO_4$ (pH 7.4) and resuspended in 0.25 M sucrose. In the experiment with $MgCl_2$ 0.2 mg mitochondrial protein was present; in the other experiments 1.1 mg. The values given are calculated for the original amount of mitochondrial protein (46 mg).

Oligomycin (μM)	*$MgCl_2$ (mM)*	*Total ADP–ATP exchange activity (μmoles [8-^{14}C]ATP per min)*		
		Mitochondria	*Extracted mitochondria*	*Supernatant*
0	10	26.4	18.2	7.6
0	0	2.8	2.7	—
10	0	0.1	0.1	—

TABLE V

EFFECT OF THE SOLUBLE ADP–ATP EXCHANGE ENZYME, F_1 AND OLIGOMYCIN ON THE P/O RATIO OF 'A' PARTICLES

20 mM potassium phosphate (pH 7.4), 5 mM Tris–sulphate (pH 7.4), 2 mM $MgSO_4$, 0.5 mM EDTA, 1 mM ADP, 30 mM glucose, 200 Cori units hexokinase, 1 mg bovine serum albumin, 20 mM succinate and 1.5 mg A particles. Temp., 25°. Reaction time, 22 min. In Expt. 2 0.2 μg oligomycin was also present.

Expt. No.	*Additions*	*ΔO (μatoms)*	*Δ hexose monophosphate (μmoles)*	*P/O*
1	None	10.7	0.30	0.03
	Exchange enzyme (50 μg)	11.4	0.22	0.02
	F_1 (120 μg)	10.7	0.79	0.07
2	None	10.4	2.65	0.25
	Exchange enzyme (50 μg)	10.7	2.17	0.21
	F_1 (120 μg)	10.4	3.85	0.37

no effect on the P/O ratio. The respiratory rate was inhibited, but this was raised to the original value by addition of cytochrome *c*.

These results of KEMP are in contrast to those of WADKINS AND LEHNINGER[8], who observed after ammonium sulphate extraction of the mitochondria a lowering of the P/O ratio, which was restored to its original value on subsequent addition of the ADP–ATP exchange enzyme. In our experiments, addition of the ADP–ATP exchange enzyme to salt-extracted mitochondria did not affect the P/O ratio. Furthermore, the soluble exchange enzyme, either that present in the crude extract or the purified preparation, had no effect on the P/O ratio (Table V) or on the ATP–P_i exchange of A particles[22] under conditions in which F_1 and low concentrations of oligomycin are active. The ADP–ATP exchange enzyme also had no effect on the ATP-driven reduction of $NADP^+$ by NADH in the KEILIN AND HARTREE heart-muscle preparation.

DISCUSSION

In agreement with WADKINS AND LEHNINGER[6,8] we have isolated from liver mitochondria an ADP–ATP exchange enzyme. It appears to be a nucleoside diphosphate kinase, that is rather specific for the adenine nucleotides. An enzyme with similar properties has been isolated from beef heart by VIGNAIS and co-workers[29,30]. The heart enzyme appears to be rather less specific and to have a lower specific activity than the liver enzyme. A coupling factor specific for Site II, CF_{II}, isolated by BEYER[31,32] also has nucleoside diphosphate kinase as its most important enzymic activity. Both the enzyme isolated by VIGNAIS and co-workers[29,30] and our preparation are inhibited by high ADP concentrations and stimulated by cytochrome *c*. However, this stimulation was obtained at all concentrations of ADP, without relief of the inhibition by ADP as reported by GLAZE AND WADKINS[13]. The explanation of this discrepancy is not clear.

One of the arguments brought forward by WADKINS AND LEHNINGER[12] in favour of a role of the ADP–ATP exchange enzyme in oxidative phosphorylation was the recombination of the soluble enzyme with digitonin particles, after which the soluble enzyme becomes partly sensitive to 2,4-dinitrophenol. However, in our opinion, inhibition of the exchange reaction by 2,4-dinitrophenol is not a reliable criterion for the participation of this reaction in oxidative phosphorylation. As can be seen from Fig. 3, the dinitrophenol-sensitive ADP–ATP exchange activity in digitonin particles increases with increasing $[Mg^{2+}]$, whereas the oligomycin-sensitive activity remains constant. In the absence of added Mg^{2+} the activity is almost completely sensitive to both oligomycin and dinitrophenol, strongly suggesting that, under these conditions, the exchange activity is related to oxidative phosphorylation. On the addition of Mg^{2+}, other enzymes insensitive to oligomycin, and therefore presumably not related to oxidative phosphorylation, but which catalyse an ADP–ATP exchange, are activated. The partial sensitivity, in the presence of Mg^{2+}, of this exchange activity to 2,4-dinitrophenol is probably due to ADP formed by the 2,4-dinitrophenol-induced ATPase. This will cause dilution of the radioactivity in the ADP. Moreover, the nucleoside diphosphate kinase, either originally present in the digitonin particles or added, is inhibited by high concentrations of ADP. That this is an adequate explanation of the sensitivity to dinitrophenol of the purified exchange

enzyme when added to the digitonin particles is supported by the experiments in which ATP was converted to ADP by adding glucose *plus* hexokinase or F_1, which are very convenient for this purpose, since they have no ADP–ATP exchange activity under these conditions. The insensitivity to oligomycin of exchange enzyme added to digitonin particles shows that the enzyme is not reincorporated into the system of oxidative phosphorylation, as supposed by WADKINS AND LEHNINGER[13].

When the enzyme is extracted from rat-liver mitochondria with 0.3 M $(NH_4)_2SO_4$ much exchange activity is solubilized. If this exchange activity represents the last step in oxidative phosphorylation, one would expect the oligomycin-sensitive exchange in the mitochondria to be lowered, but this was not found. It must be concluded that the extracted enzyme activity is not derived from the oligomycin-sensitive ADP–ATP exchange. The results of KEMP (unpublished) are in agreement with this finding. We could find no 'coupling factor' activity of the ADP–ATP exchange enzyme when tested with salt-extracted mitochondria (contrast ref. 8) or with A particles under conditions in which other coupling factors are active.

In summary, the ADP–ATP exchange enzyme of WADKINS AND LEHNINGER is a nucleoside diphosphate kinase, that can be easily extracted[33] from the mitochondria and that is rather specific for the adenine nucleotides. A possible role of this enzyme in oxidative phosphorylation, as suggested by WADKINS AND LEHNINGER, is, in the light of our results, improbable.

ACKNOWLEDGEMENTS

The authors wish to thank Professor E. C. SLATER for his interest and advice, Dr. R. P. GLAZE for giving us the details of the isolation procedure prior to publication, and Miss J. H. DE KONING for skilful technical assistance. This work was supported in part by grants from the Life Insurance Medical Research Fund and the U.S. Public Health Service (Grant AM-08690).

REFERENCES

1 C. L. WADKINS, *J. Biol. Chem.*, 236 (1961) 221.
2 C. L. WADKINS, *Biochem. Biophys. Res. Commun.*, 7 (1962) 70.
3 F. HUIJING AND E. C. SLATER, *J. Biochem. Tokyo*, 49 (1961) 493.
4 R. J. GUILLORY AND E. C. SLATER, *Biochim. Biophys. Acta*, 105 (1965) 221.
5 F. L. BYGRAVE AND A. L. LEHNINGER, *J. Biol. Chem.*, 241 (1966) 3894.
6 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 233 (1958) 1589.
7 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 238 (1963) 2555.
8 C. L. WADKINS AND A. L. LEHNINGER, *Federation Proc.*, 22 (1963) 1092.
9 C. COOPER AND R. G. KULKA, *J. Biol. Chem.*, 236 (1961) 2351.
10 R. G. KULKA AND C. COOPER, *J. Biol. Chem.*, 237 (1962) 936.
11 J. R. BRONK AND W. W. KIELLEY, *Biochim. Biophys. Acta*, 29 (1958) 369.
12 C. L. WADKINS AND A. L. LEHNINGER, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 1576.
13 R. P. GLAZE AND C. L. WADKINS, *Biochem. Biophys. Res. Commun.*, 15 (1964) 194.
14 C. L. WADKINS AND R. P. GLAZE, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Vol. 2, Wiley, New York, 1965, p. 1018.
15 M. E. PULLMAN, H. S. PENEFSKY, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3322.
16 H. S. PENEFSKY, M. E. PULLMAN, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3330.
17 H. ZALKIN, M. E. PULLMAN AND E. RACKER, *J. Biol. Chem.*, 240 (1965) 4011.
18 F. L. CRANE, J. L. GLENN AND D. E. GREEN, *Biochim. Biophys. Acta*, 22 (1956) 475.
19 C. J. O. R. MORRIS AND P. MORRIS, *Separation Methods in Biochemistry*, Pitman, London, 1964, p. 138.
20 D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.

21 T. M. DEVLIN AND A. L. LEHNINGER, *J. Biol. Chem.*, 233 (1958) 1586.
22 J. M. FESSENDEN AND E. RACKER, *J. Biol. Chem.*, 241 (1966) 2483.
23 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
24 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
25 E. C. SLATER, *Biochem. J.*, 53 (1953) 521.
26 R. B. DUFFIELD AND M. CALVIN, *J. Am. Chem. Soc.*, 68 (1946) 557.
27 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 39 (1945) 289.
28 E. MARGOLIASH, *Biochem. J.*, 56 (1954) 529; 535.
29 J. LATURAZE AND P. V. VIGNAIS, *Biochim. Biophys. Acta*, 92 (1964) 184.
30 M. G. COLOMB, J. G. LATURAZE AND P. V. VIGNAIS, *Biochem. Biophys. Res. Commun.*, 24 (1966) 909.
31 R. E. BEYER, *Biochem. Biophys. Res. Commun.*, 16 (1964) 460.
32 R. E. BEYER, *Biochem. Biophys. Res. Commun.*, 17 (1964) 764.
33 M. KLINGENBERG AND E. PFAFF, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 180.