Properties of an Oligomycin-sensitive Adenosine Diphosphate-Adenosine Triphosphate Exchange Reaction in Intact Beef Heart Mitochondria*

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SUMMARY

Intact beef heart mitochondria having high acceptor control ratios catalyze an oligomycin-sensitive adenosine diphosphate-ATP exchange reaction in the presence of 6.0 mM Mg++ at a rate of about 400 to 600 mmolates per min per mg of mitochondrial protein, or somewhat higher than the rate of oxidative phosphorylation in beef heart mitochondria. The oligomycin-sensitive component of the total ADP-ATP exchange activity is saturated with Mg++ at much lower concentrations (~1.0 mM) than the oligomycin-insensitive component (~8.0 mM). Gramicidin, vallinomycin, aurovertin, azide, arsenate, and atracyloside inhibit the ADP-ATP exchange, to an extent not exceeding the inhibition by oligomycin, whereas the adenylate kinase activity is insensitive to these agents.

Beef heart mitochondria also catalyze uridine diphosphate-UTP, cytidine diphosphate-CTP, guanosine diphosphate-GTP, and ADP-dCTP exchanges, but these are insensitive to oligomycin and occur at less than 10% of the rate of the ADP-ATP exchange. Freezing and thawing of beef heart mitochondria, which has been reported not to cause loss of oxidative phosphorylation as measured by the P:O ratio, caused loss of the oligomycin-sensitive ADP-ATP exchange, loss of acceptor control of respiration, and gain of ATPase activity. It is suggested that the loss of oligomycin-sensitive ADP-ATP exchange activity during preparation of phosphorylating submitochondrial particles is caused by a molecular transformation of the ATP-synthesizing system so that the affinity for ADP is decreased, with a resulting gain of ATPase activity.

Phosphorylating particles prepared from rat liver mitochondria catalyze an ADP-ATP exchange reaction that is inhibited by 2,4-dinitrophenol (1–12). This reaction is apparently associated with oxidative phosphorylation, since intact mitochondria isolated from a variety of animal tissues have considerable dinitrophenol- and oligomycin-sensitive ADP-ATP exchange activity (13–16). ADP-ATP exchange reactions, sometimes sensitive to uncoupling agents, have also been found in microorganisms (17, 18) and in preparations derived from chloroplasts (19–21). On the basis of the sensitivity of the ADP-ATP exchange reaction in mitochondria to both 2,4-dinitrophenol and oligomycin, as well as other properties summarized elsewhere (cf. 2, 4–6, 15, 22, 23), it has been postulated that the exchange is catalyzed by an enzyme or enzymes normally participating in oxidative phosphorylation, presumably in the terminal steps leading to ATP formation (2, 12, 22, 23).

Recent studies on the activity and properties of submitochondrial particles and coupling factors derived from beef heart mitochondria are somewhat conflicting with regard to this hypothesis. Wadkins and Lehninger (15) reported a few data on intact beef heart mitochondria showing that they catalyze an oligomycin-sensitive ADP-ATP exchange reaction at a high rate. Supporting this observation is the report of Beyer (24) that the highly purified coupling factor (ATP synthetase) for Site II, also isolated from beef heart mitochondria, catalyzes an ADP-ATP exchange reaction. On the other hand, the soluble beef heart mitochondrial ATPase of Pullman et al. (25), which can restore oxidative phosphorylation in pretreated beef heart mitochondria (26, 27), was reported to show no detectable ADP-ATP exchange activity. Similarly, the beef heart coupling factors described by Sanadi and his colleagues (28–30) also have been found to lack ADP-ATP exchange activity.1 Differences between rat liver and beef heart are also evident in studies of membrane fragments having electron transport activity. Whereas submitochondrial particles from liver have considerable dinitrophenol-sensitive ADP-ATP exchange activity (2, 4, 5, 7, 8), Zalkin et al. (31) have reported that submitochondrial particles derived from beef heart are nearly devoid of such activity.

Because these findings suggested that there may be some significant differences in the stability or ease of dissociation of the enzymes involved in oxidative phosphorylation in the two

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1 C. L. Wadkins, personal communication.
types of mitochondria, it appeared desirable to carry out a more
detailed study of the oligomycin-sensitive ADP-ATP exchange
reaction in beef heart mitochondria. This communication reports the results of this study and a comparison of the behavior of
the ADP-ATP exchange in beef heart and rat liver mito-
chondria.

EXPERIMENTAL PROCEDURES

Mitochondria were isolated from fresh beef heart by the
Nagarse method of Hasefi, Juretschuk, and Hasvik (32); they were washed twice with 0.25 M sucrose with special care to remove the fluffy layer. The acceptor control ratio (i.e. the ratio of State 3 to State 4 respiratory rates in the presence of pyruvate plus malate as substrate) was determined with the Clark oxygen electrode as described by Chance and Williams (32). Thrice preparations of mitochondria having acceptor control ratios less
than 4.0 were discarded.

Protein was determined by the biuret reaction with crystal-

cized egg albumin as standard.

The ADP-ATP exchange reaction was assayed precisely as
previously described (15); all components of the exchange assay
except ATP and 14C-ADP were contained in a total volume of
0.5 ml and were preincubated for 8 min at 30°. ATP and 14C-
ADP in a total volume of 0.1 ml were then added to initiate the
reaction. After 10 min of incubation, the reactions were stopped
by the addition of 0.1 ml of 2.5 M perchloric acid, and the precipi-
tate was removed by centrifugation. The supernatant solution was
then neutralized with an equivalent amount of KOH. After
separation of the precipitated potassium perchlorate by cen-
trifugation, 0.05-ml aliquots of the supernatant solution were
subjected to high-voltage electrophoresis (15). The adenine
nucleotide spots were located under ultraviolet light, eluted with
water, plated, and the radioactivity was determined in a low
radioactivity (34, 35). It is also about the same as that required to
inhibit oxidative phosphorylation in beef heart submitochondrial
preparations (36).

The ADP-ATP exchange reaction was calculated
from the equation used by Wadkins and Lehninger (15), which
corrects for the change in specific activity of 14C-ADP as the
system approaches isotopic equilibrium. In all assays of the
ADP-ATP exchange, care was taken to measure initial reaction
dates to minimize errors due to approach to isotopic equilibrium
and the effects of the changing point of equilibrium when ATPase
activity is occurring simultaneously. Separation of UDP from
UTP, GDP from GTP, and CDP from CTP was carried out exactly as described for ADP and ATP. It was necessary to
increase the time of electrophoresis in the case of GDP-GTP by
about 30 min.

Adenylate kinase activity was measured in two kinds of assay
systems, by determining the conversion of 14C-labeled AMP to
14C-labeled ADP in the presence of unlabeled ATP (i.e. the
forward direction) or by determining the conversion of 14C-
labeled ADP to labeled AMP and labeled ATP (the reverse
direction); details are given in the legends. ATPase activity
was estimated by colorimetric determination of inorganic phospho-

date formed during the reaction. It was also checked by
determination of the changes in the concentration of ATP and
ADP measured by paper electrophoresis.

RESULTS

Sensitivity of ADP-ATP Exchange Activity to Oligomycin—
When assayed in the absence of added Mg++, conditions in which
the oligomycin-sensitive component of the total ADP-ATP exchange
activity of intact rat liver mitochondria is best ob-

served (15), intact beef heart mitochondria with a high respira-

tory control ratio showed considerable ADP-ATP exchange activity with a high degree of sensitivity to oligomycin (Table I;
Fig. 1). The experiment in Fig. 1 shows that the exchange
reaction is completely inhibited at about 0.7 μg of oligomycin per
mg of mitochondrial protein; half-maximum inhibition was
given by 0.1 μg of oligomycin per mg. The amounts of oligomycin
required are thus about the same as those required to inhibit
the ADP-ATP exchange reaction of rat liver mitochondria (14),
as well as phosphorylating electron transport and ATPase ac-


tivity (34, 35). It is also about the same as that required to
inhibit oxidative phosphorylation in beef heart submitochondrial
preparations (36).

**Table I**

**ADP-ATP exchange activity in fresh intact beef heart mitochondria**

The test system contained 10.6 mM Tris-chloride (pH 7.4), 83 mM sucrose, 2.0 mM 1C-ADP, 6.0 mM ATP, and 150 μg of mitochon-
drial protein in a total volume of 0.60 ml. Where indicated, 6.0 mM MgCl2, 0.85 μm 2,4-dinitrophenol, and 1.0 μg of oligomycin were
added. All assays were carried out for 10 min at 30°.
The data in Table I suggest that there are at least two components of the total ADP-ATP exchange activity; one that is essentially completely inhibited by oligomycin and by 2,4-dinitrophenol, but is less dependent on added Mg++ for activity, and a second component that is not sensitive to oligomycin but is completely dependent on added Mg++ for activity. The ADP-ATP exchange assays in the absence of added Mg++ was found to vary considerably from preparation to preparation; this variation may be a reflection of variable amounts of intramitochondrial Mg++ that are available for activation of the enzyme. The total oligomycin-sensitive ADP-ATP exchange activity assayed in the presence of excess Mg++, but in the absence of phosphate (see below), is in the range of 220 to 380 mmoles of ADP exchanged per mg of mitochondrial protein per min. This is substantially higher than the activity given by rat liver mitochondria, but somewhat less than the activity of rat kidney mitochondria (15).

Relative Amounts of Oligomycin-sensitive and -insensitive ADP-ATP Exchange Activity—Data collected in Table I show the sensitivity of the ADP-ATP exchange in freshly prepared beef heart mitochondria to oligomycin and 2,4-dinitrophenol in the absence and presence of Mg++. When the assay was carried out in the absence of added Mg++, the exchange reaction was inhibited over 90% by oligomycin and at least 85% by 2,4-dinitrophenol in all preparations tested, in agreement with the results reported earlier by Wadkins and Lehninger (15). When 6.0 mM Mg++ was added, there was a 3- to 5-fold increase in the rate of the total observed ADP-ATP exchange, but only 50 to 70% of the additional activity evoked by added Mg++ was found to be sensitive to oligomycin; thus, approximately 50 to 60% of the total ADP-ATP exchange activity observed in the presence of 6.0 mM Mg++ was oligomycin-sensitive. This finding qualitatively resembles the data on the ADP-ATP exchange in other types of mitochondria (15), but differs quantitatively from the few data on beef heart mitochondria reported earlier (15). The latter data indicated that the Mg++-stimulated ADP-ATP exchange activity was fully inhibited by oligomycin, rather than some 50 to 60% as found in the present study. However, the earlier data were obtained from experiments in which 1.0 mM Mg++ was present,2 whereas the experiments reported here were carried out at 6.0 mM Mg++. The significance of this point will be developed below.

Components of the total ADP-ATP exchange activity; one that is essentially completely inhibited by oligomycin and by 2,4-dinitrophenol and is less dependent on added Mg++ for activity, and a second component that is not sensitive to oligomycin but is completely dependent on added Mg++ for activity. The ADP-ATP exchange activity assayed in the absence of added Mg++ was found to vary considerably from preparation to preparation; this variation may be a reflection of variable amounts of intramitochondrial Mg++ that are available for activation of the enzyme. The total oligomycin-sensitive ADP-ATP exchange activity assayed in the presence of excess Mg++, but in the absence of phosphate (see below), is in the range of 220 to 380 mmoles of ADP exchanged per mg of mitochondrial protein per min. This is substantially higher than the activity given by rat liver mitochondria, but somewhat less than the activity of rat kidney mitochondria (15).

Maximum activity of the oligomycin-sensitive ADP-ATP exchange reaction in rat liver mitochondria and submitochondrial preparations is known to be in the presence of inorganic phosphate (10, 15). As will be shown below, the addition of 10 mM phosphate approximately doubles the oligomycin-sensitive activity of the ADP-ATP exchange in beef heart mitochondria to values in the range 500 to 750 mmoles per mg of protein per min. This value may now be compared with the rate of respiration of beef heart mitochondria (substrate, pyruvate + malate), namely, about 100 to 200 mmoles of oxygen uptake per mg per min (see Table I). The ADP-ATP exchange reaction in beef heart mitochondria thus proceeds at a rate that exceeds on a molar basis the net rate of oxidative phosphorylation, i.e. 300 to 600 mmoles of ATP formed per mg per min, assuming a 1:0 ratio of 3.0 for pyruvate + malate.

Mg++ Requirement of Oligomycin-sensitive and -insensitive ADP-ATP Exchanges—The oligomycin-sensitive component of the total ADP-ATP exchange activity was found to require a much lower concentration of added Mg++ for full activation than the oligomycin-insensitive component, as is shown by the data in Fig. 2. Although the exact concentration of Mg++ required for half-maximal stimulation of the oligomycin-sensitive component cannot be derived from the points given, it is evidently less than 0.3 mM; full saturation was achieved at about 1.0 mM Mg++. On the other hand, Fig. 2 shows that the oligomycin-insensitive component was not fully saturated at 1.0 mM nor even at 6.0 mM MgCl2. In other experiments it was found that about 8.0 mM Mg++ was required for full saturation of the oligomycin-insensitive component. These data clearly suggest that the oligomycin-sensitive and insensitive components of the ADP-ATP exchange may be catalyzed by different enzymes; the oligomycin-sensitive reaction is saturated with Mg++ at concentrations far below (<1.0 mM) those required to form complexes with the adenine nucleotide substrates added (total concentration in system, 8 mM), whereas the oligomycin-insensitive reaction is saturated with Mg++ at such concentrations (~0.8 mM) that the adenine nucleotides are probably fully complexed.

Another important point may be derived from the data in Fig. 2. It will be noted that the fraction of the total ADP-ATP exchange activity that is sensitive to oligomycin varies with the Mg++ concentration. At 1.0 mM Mg++, the concentration used in the survey of different mitochondria by Wadkins and Lehninger (15), about 90% of the total activity was oligomycin-sensitive. However, at 6.0 mM Mg++, the concentration used in most experiments of the present study, only about 50% of the total activity is oligomycin-sensitive. It is evident that assays carried out at 1.0 mM Mg++ do not measure the full activity of the oligomycin-insensitive component of the total ADP-ATP exchange activity. It is therefore likely that the data reported earlier indicating that the Mg++-stimulated ADP-ATP exchange activity is almost completely sensitive to oligomycin in beef heart, rat heart, and rat kidney mitochondria.
(15) were collected under conditions in which the oligomycin-insensitive component was not completely saturated with 

**Effect of ADP and ATP Concentration on Oligomycin-sensitive Exchange.**—Data in Fig. 3 show the effect of nucleotide concentration on the rate of the oligomycin-sensitive ADP-ATP exchange reaction of beef heart mitochondria assayed in the absence of added Mg++. It is seen that the activity was maximal at concentrations of about 2.0 mM ADP when ATP concentration was 6.0 mM; the extrapolated $K_m$ for ADP is about 0.5 mM. When ADP was held constant at 2.0 mM, the exchange rate was maximal at about 5.0 mM ATP, with an extrapolated $K_m$ of about 1.5 mM ATP. These results may be compared with $K_m$ figures of 1.0 mM for ADP and of 0.3 mM for ATP in the case of rat liver mitochondria (15).

**Kinetics and Approach to Equilibrium.**—The ADP-ATP exchange reaction in beef heart mitochondria was found to proceed rapidly to complete isotopic equilibrium in the absence or presence of added Mg++. Equilibrium was reached much sooner when Mg++ was present. In the absence of added Mg++, oligomycin inhibited the total exchange activity most at the initial stages. Since the oligomycin-insensitive component of the exchange continued in a nearly linear fashion for an extended period, it is evident that oligomycin sensitivity of the ADP-ATP exchange must be measured in the initial stages of the exchange reaction, as was earlier shown for the case of rat liver mitochondria (15). Failure to observe oligomycin sensitivity of the ADP-ATP exchange, such as was reported by Huijing and Slater (35), may be caused if measurements are made near or at the point of isotopic equilibrium.

The data in Fig. 4 also show the actual amounts of ATP and ADP present in the systems at the beginning and at the end of the reaction periods. In the absence of added Mg++, about 13% of the ATP added underwent hydrolysis to ADP, but when oligomycin was also present, less than 10% underwent hydrolysis. In the presence of added Mg++, 18% of the added ATP was hydrolyzed. It has already been shown (4, 15, 15) that changes of this order of magnitude in the ATP-ADP ratio during the exchange assay do not influence the measurement of the exchange rate under the specific conditions employed; the concentrations of ATP and ADP remaining still exceed those required for quantitative measurement of the exchange reaction.

**Other Properties of ADP-ATP Exchange.**—When unlabeled ATP was omitted from the test systems, no detectable labeled ATP was formed from 14C-labeled ADP in the absence of added Mg++. It is evident that the formation of labeled ATP is dependent on the presence of Mg++. Since the maximum rate of oxygen uptake under the conditions of the exchange assay (no added substrate or Pi) was of the order of 9 mmoles per min per mg of protein, the maximum amount of Pi that can be esterified at the expense of endogenous respiration is less than 27 mmoles per min per mg (assuming a P:O ratio of 3.0), or about 10 to 15% of the exchange activity usually obtained. These observations eliminate the possibility that the formation of 14C-ATP in the exchange reaction assay system is the consequence of continuous oxidative phosphorylation of labeled ADP to ATP at the expense of endogenous respiratory substrates, accompanied by continuous hydrolysis of ATP to ADP. The oligomycin-sensitive component of the ADP-ATP exchange reaction was found to be dependent on the potency of the medium; the rate was highest in a medium containing either 150 mM KCl or 83 mM sucrose.

**Insensitivity of Adenylate Kinase Activity to Dinitrophenol and Oligomycin.**—In the absence of added Mg++, beef heart mitochondria catalyzed negligible rates of adenylate kinase activity, assayed in either direction (Table II). The addition of Mg++ to the incubation system, however, evoked a large amount of adenylate kinase activity of about the same order of magnitude as the total ADP-ATP exchange activity. However, the adenylate kinase activity, when assayed in either direction, was not inhibited by either oligomycin or dinitrophenol. These findings show that intact beef heart mitochondria contain sufficient endogenous Mg++ to catalyze considerable oligomycin-sensitive

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**Fig. 2.** Effect of Mg++ concentration on oligomycin-sensitive and -insensitive components of ADP-ATP exchange. System contained 2.0 mM 14C-ADP and 6.0 mM ATP, 300 μg of mitochondrial protein, and MgCl₂ as shown in a final volume of 0.60 ml. Incubated 10 min at 30°.

**Fig. 3.** A, effect of ADP concentration on the ADP-ATP exchange. The exchange assay was carried out as described in Table I, with ATP concentration held at 6.0 mM and ADP varied. The system contained 300 μg of mitochondrial protein in a final volume of 0.60 ml. B, effect of ATP concentration on the ADP-ATP exchange. Experimental conditions were as described in A, with ADP concentration held constant at 2.0 mM and ATP varied. The system contained 300 μg of mitochondrial protein in a final volume of 0.60 ml. Incubation was for 10 min at 30°.
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MINUTES MINUTES

FIG. 4. Kinetics and equilibrium of the ADP-ATP exchange. The reaction system contained 0.0 mM ATP, 2.0 mM 14C-ADP (10,000 cpm per pmole), 83 mM sucrose, 16.6 mM Tris-chloride (pH 7.4), and 4.0 mM MgCl₂ where indicated. In the experiment shown at left, 3.6 mg of mitochondrial protein were present; at right, 1.8 mg were added in a total volume of 6.0 ml. Oligomycin was added where indicated at 1 pg per ml. The incubation was carried out at 30°. At the specified times, 0.50 ml aliquots were removed, and the specific activities of ATP and ADP were determined.

TABLE II

Effects of dinitrophenol and oligomycin on adenylate kinase activity

<table>
<thead>
<tr>
<th>Rate without Mg⁺⁺</th>
<th>6.0 mM Mg⁺⁺</th>
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<tbody>
<tr>
<td>Rate</td>
<td>Inhibition</td>
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<tr>
<td>µmoles min⁻¹ mg⁻¹</td>
<td>%</td>
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<tr>
<td>10</td>
<td>750</td>
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<tr>
<td>0</td>
<td>600</td>
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<tr>
<td>0</td>
<td>800</td>
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<tr>
<td>25</td>
<td>316</td>
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<td>0</td>
<td>340</td>
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ADP-ATP exchange activity, but insufficient Mg⁺⁺ to yield significant adenylate kinase activity. Similar results were previously obtained with intact rat liver mitochondria (13). Other evidence indicating that the ADP-ATP exchange is not catalyzed by adenylate kinase is given below.

Effect of Inorganic Phosphate on ADP-ATP Exchange, the 32P-ATP Exchange, and Adenylate Kinase Activity—It was shown earlier that inorganic phosphate stimulated the ADP-ATP exchange in intact rat liver mitochondria (15), confirming the findings of Cooper and Kulka (10) on digitonin particles of rat liver mitochondria. The data in Fig. 5 show that the ADP-ATP exchange of beef heart mitochondria was likewise markedly stimulated by increasing concentrations of inorganic phosphate, maximal stimulation being given by about 10 mM phosphate, which approximately doubled the rate. The data in Fig. 5 also show that inorganic phosphate had no effect on adenylate kinase activity. Guillory and Slater (16) recently proposed that stimulation of the ADP-ATP exchange by added inorganic phosphate in rat liver mitochondria can be accounted for by net phosphorylation of ADP in the course of the incubation, at the expense of the oxidation of endogenous respiratory substrates. However, under the conditions described here, no net increase in ATP formation was observed. The reasons for the discrepancy with the data of Guillory and Slater are not clear; however, the latter authors used 1.2 mg of mitochondrial protein per ml, whereas in the present experiments, in which no increase of ATP was observed, only 370 μg of mitochondrial protein per ml were employed. At the higher mitochondrial concentrations a
The ADP-ATP exchange assay was carried out under the conditions described in Table I. In each tube 150 μg of mitochondrial protein were present. Hydrolysis of ATP occurring during the ADP-ATP exchange assay was measured by determining the appearance of inorganic phosphate. Data are in terms of exchange or ATPase activity per vessel per 10 min at 30°C.

### Table III

<table>
<thead>
<tr>
<th>Mg++</th>
<th>Additions</th>
<th>ADP-ATP exchange activity</th>
<th>ATPase activity</th>
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<tr>
<td>mM</td>
<td></td>
<td>mpmoles</td>
<td>mpmoles</td>
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<tr>
<td>0</td>
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<td>288</td>
<td>80</td>
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<tr>
<td></td>
<td>Dinitrophenol</td>
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<td>144</td>
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<td>Oligomycin</td>
<td>0</td>
<td>72</td>
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<td>1730</td>
<td>128</td>
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<td></td>
<td>Dinitrophenol</td>
<td>970</td>
<td>200</td>
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<tr>
<td></td>
<td>Oligomycin</td>
<td>1200</td>
<td>80</td>
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</table>

larger amount of endogenous respiratory substrate may be present.

The ADP-ATP exchange activity of beef heart mitochondria, when measured under exactly the same conditions as used for the assay of the ATP-ADP exchange, was found to be stimulated in a similar manner by increasing concentrations of inorganic phosphate (Fig. 5). The ADP-ATP exchange was completely inhibited by oligomycin.

### Table IV

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>ADP-ATP exchange</th>
<th>ATPase activity</th>
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<td>No Mg++</td>
<td>2.5 mM Mg++</td>
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<td>Experiment 1</td>
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<td>Experiment 2</td>
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Effect of Other Inhibitors on ADP-ATP Exchange—As is shown in Table IV the ADP-ATP exchange reaction assayed in the presence of Mg++, addition of dinitrophenol produced only a slight additional ATPase activity, to about 210 mpmoles, whereas about 760 mpmoles of ADP-ATP exchange activity were inhibited by the dinitrophenol.
Oligomycin-sensitive ADP-ATP Exchange Reaction in Mitochondria

The assay system contained 10.6 mM Tris-chloride (pH 7.4), 83 mM sucrose, 6.0 mM ATP (or other nucleoside 5'-triphosphates indicated), 2.0 mM 14C-labeled ADP (or other nucleoside 5'-diphosphates), and 6.0 mM MgCl₂ as shown. The final volume was 0.60 ml; 1.0 μg of oligomycin was added as shown. Each vessel contained 300 μg of mitochondrial protein and was incubated for 10 min at 30°C.

**Table V**

<table>
<thead>
<tr>
<th>Nucleotide system</th>
<th>Nucleotide exchange rate</th>
<th>6.0 mM Mg++</th>
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<tr>
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<td>Without oligomycin</td>
<td>With oligomycin</td>
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<tr>
<td></td>
<td>Without Mg++</td>
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<tr>
<td>ADP-ATP</td>
<td>144</td>
<td>0</td>
</tr>
<tr>
<td>ADP-dCTP</td>
<td>11</td>
<td>5</td>
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<tr>
<td>CDP-CTP</td>
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<td>GDP-CTP</td>
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<td>UDP-UTP</td>
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**Table VI**

<table>
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<tr>
<th>Mg++</th>
<th>Additions</th>
<th>ADP-ATP exchange activity</th>
<th>mmoles 14C-CTP min⁻¹ mg⁻¹</th>
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<td>Oligomycin</td>
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<td>15</td>
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<td>556</td>
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<td></td>
<td>Oligomycin</td>
<td>377</td>
<td>426</td>
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° Acceptor control ratio: Experiment 1, 1.2; Experiment 2, 1.1.

The exchange assay was carried out as described in Table I. Mitochondria were isolated from fresh unfrozen beef hearts by the method of Crane et al. (47). Concentrations of mitochondrial protein were 3 mg and 160 μg, respectively (Experiment 1), and 0.1 mg and 280 μg, respectively (Experiment 2), in the absence and presence of added Mg++ ions. Dinitrophenol was added at 0.85 mM and oligomycin at 1.0 μg per ml. The assay was carried out at 30°C for 10 min.

Activity of intact beef heart mitochondria was found not to be inhibited significantly by any of these agents.

The ADP-ATP exchange was inhibited by HCN in the presence of succinate + β-hydroxybutyrate, conditions which also inhibit ADP-ATP exchange and ATPase activity in rat liver mitochondria, presumably through maintenance of the completely reduced state of the respiratory chain and pyridine nucleotides (3).

Other Nucleoside Diphosphate-Nucleoside Triphosphate Exchange Reactions—Data in Table V show that in the absence of added Mg++, conditions under which considerable oligomycin-sensitive ADP-ATP exchange activity may be observed, fresh beef heart mitochondria failed to catalyze significant rates of exchange between 14C-UDP and UTP, 14C-ADP and dCTP, 14C-CDP and CTP, or 14C-GDP and GTP, indicating the specificity for both ADP and ATP in the oligomycin-sensitive nucleotide exchange reaction.

Addition of Mg++ did evoke some CDP-CTP, UDP-UTP, and GDP-GTP exchange activity. However, these activities were less than 10% of the rate of the ADP-ATP exchange and were insensitive to oligomycin. The failure of the GDP-GTP exchange to be inhibited by oligomycin or dinitrophenol may be relevant to the mechanism of the oligomycin sensitivity of the substrate level phosphorylation linked to oxidation of α-ketoglutarate (cf. Davis (40)).

Respiratory Control and Integrity of Mitochondrial Structure in Relation to ADP-ATP Exchange Reactions—The oligomycin-inhibited component of the ADP-ATP exchange activity in beef heart mitochondria is extremely sensitive to treatments leading to loss of respiratory control. Beef heart mitochondria prepared by the Nagarse method (32) and employed in this study showed not only high rates of oligomycin-sensitive ADP ATP exchange, but also high respiratory control ratios. On the other hand, beef heart mitochondria prepared by the blender method of Crane et al. (47), which in our hands showed little or no acceptor control of respiration, were found to have no oligomycin-sensitive exchange activity.

**Table VII**

<table>
<thead>
<tr>
<th>Activity measured</th>
<th>Additions</th>
<th></th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>Frozen</td>
<td>mmoles 14C-CTP min⁻¹ mg⁻¹</td>
</tr>
<tr>
<td>Acceptor control ratio</td>
<td>None</td>
<td>608</td>
<td>349</td>
</tr>
<tr>
<td>ADP-ATP exchange</td>
<td>None</td>
<td>277</td>
<td>393</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>None</td>
<td>533</td>
<td>441</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>None</td>
<td>504</td>
<td>454</td>
</tr>
<tr>
<td>ATPase</td>
<td>None</td>
<td>490</td>
<td>532</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>None</td>
<td>370</td>
<td>383</td>
</tr>
</tbody>
</table>

absence and presence of added Mg++ was inhibited not only by 2,4-dinitrophenol and oligomycin, but also by valinomycin and gramicidin, inhibitors of oxidative phosphorylation that apparently act by permitting K+ and Na++ either to cross the mitochondrial membrane or membranes or to interact with high energy intermediates (37, 38). The ADP-ATP exchange was also inhibited by azide, arsenate, and aurovertin, a specific inhibitor of oxidative phosphorylation (39). The effects of gramicidin, azide, and arsenate agree with earlier tests on rat liver mitochondria (13, 15). Atractyloside, which blocks ATP-dependent reactions in intact mitochondria (40-45) except adenylate kinase activity (42), was also found to inhibit the ADP-ATP exchange reaction. In contrast, adenylate kinase activity is not inhibited significantly by any of these agents.
exchange activity (Table VI). "Blender" mitochondria showed only traces of ADP-ATP exchange activity in the absence of added Mg++, whereas "Nagarse" mitochondria have considerable exchange activity in the absence of added Mg++, nearly all of which is inhibited by oligomycin (Table I). When Mg++ was added, blender mitochondria showed considerable ADP-ATP exchange activity, but none of the Mg++-stimulated activity was inhibited by oligomycin or dinitrophenol. However, it is significant that blender mitochondria showed about as much Mg++-stimulated oligomycin-insensitive activity as Nagarse mitochondria.

Beef heart mitochondria prepared by the Nagarse method were found to lose from 60 to 75% of their total ADP-ATP exchange activity following a cycle of freezing and thawing. Essentially none of the remaining activity was sensitive to oligomycin or dinitrophenol. Data of a typical experiment are given in Table VII. In this experiment, freezing and thawing caused a decline of the acceptor control ratio from 4.2 to about 1.2. In this experiment it will be noted that the loss of oligomycin-sensitive exchange activity that occurred on freezing and thawing was accompanied by appearance of ATPase activity. Adenylate kinase suffered less than an 8% loss in activity in the freezing-thawing cycle; the parallel stability of adenylate kinase activity and of the oligomycin-insensitive ADP-ATP exchange activity is noteworthy. Assays carried out in the absence of added Mg++ also showed that the oligomycin-sensitive ADP-ATP exchange activity was completely lost after freezing and thawing. Direct tests showed that the loss of activity on freezing and thawing was not a matter of leakage of the ADP-ATP exchange activity into the suspending medium.

### DISCUSSION

The data reported in this paper show that the oligomycin- and dinitrophenol-sensitive component of the ADP-ATP exchange activity of intact beef heart mitochondria is similar in most respects to the reaction in intact rat liver mitochondria (13, 15). Closely similar are the relative rates of the oligomycin-sensitive and -insensitive components of the total exchange activity, the dependence of the oligomycin-sensitive exchange activity on the concentration of ATP, ADP, and Mg++, the stimulation of the oligomycin-sensitive exchange by phosphate, the response of the oligomycin-sensitive exchange to inhibitors of oxidative phosphorylation and to reduction of the respiratory chain, and its sensitivity to treatments causing loss of respiratory control. The apparent differences between phosphorylating submitchondrial particles from beef heart and rat liver must, therefore, be a function of the response of the enzymes catalyzing the oligomycin-sensitive exchange in the two types of mitochondria to the procedures used to disrupt mitochondrial structure. This point will be considered in more detail below.

This paper also provides additional evidence beyond that already reported (1-7, 12, 13, 15, 22, 23) that the oligomycin-sensitive portion of the total ADP-ATP exchange activity is catalyzed by an enzyme participating in oxidative phosphorylation. First, the rate of the oligomycin-sensitive component of the ADP-ATP exchange of intact beef heart mitochondria was found to be at least as high as the rate of net oxidative phosphorylation. Second, the oligomycin-sensitive component of the total ADP-ATP exchange activity in beef heart mitochondria is sensitive not only to 2,4-dinitrophenol, gramicidin, and aro-
insensitive component of the ADP-ATP exchange, which resembles adenylate kinase activity in requiring a high Mg\(^{2+}\) concentration for saturation, in its stability to freezing and thawing or blender treatment, its failure to be stimulated by inorganic phosphate, and its insensitivity to dinitrophenol, atracyloside, aurovertin, valinomycin, gramicidin, azide, and arsenate. Furthermore, the oligomycin-insensitive component of the ADP-ATP exchange activity has about the same rate as the adenylate kinase activity.

It is of some interest that intact beef heart mitochondria show very high ADP-ATP exchange activity and no UDP-UTP, GDP-GTP, or GDP-CTP exchange activity when assayed in the absence of Mg\(^{2+}\). Even in the presence of added Mg\(^{2+}\), the other nucleotide exchanges have only 10% or less of the activity of the ADP-ATP exchange. It appears possible that this apparent specificity of the exchange for adenine nucleotides is brought about by the fact that nucleotides other than ADP or ATP cannot enter intact mitochondria readily; the atracyloside-sensitive exchange-diffusion pathway for entry of ADP or ATP appears to be specific for adenine nucleotides (48, 50). On the other hand, submitochondrial particles prepared by sonic or cholate treatment are known to utilize not only ADP, but other nucleoside 5'-diphosphates as phosphate acceptors (48), presumably because the coupling enzymes are intrinsically nonspecific and because damage to the selectivity of the membrane allows access of all the nucleoside 5'-diphosphates. The ATPase (coupling factor F\(_1\)) isolated from beef heart mitochondria by Pullman et al. (25) has been found to act not only on ATP, but also on ITP, GTP, and UTP; presumably it is also able to utilize IDP, GDP, and UDP as phosphate acceptors when it is functioning as a coupling factor for oxidative phosphorylation. The nucleoside diphosphokinase isolated by Chiga and Plaut (51) from mitochondria, which may also be related to oxidative phosphorylation, also acts on nucleotides other than those of adenine. Elsewhere evidence will be presented that the purified ADP-ATP exchange enzymes of beef liver and beef heart mitochondria also catalyze exchanges between other homologous nucleotide pairs.

As in the case of rat liver mitochondria, the oligomycin-sensitive component of the ADP-ATP exchange has maximal activity in beef heart mitochondria when they are most intact and still possess a high degree of acceptor control of respiration. Freezing and thawing or blender treatment caused loss of nearly all the oligomycin-sensitive ADP-ATP exchange activity of beef heart mitochondria. Freezing and thawing are tolerated by beef heart mitochondria, which still show nearly maximal P:O ratios after this procedure (52). In fact, beef heart mitochondria are often stored in the frozen state before use as starting material for reconstitution experiments (31, 52). However, since frozen-thawed beef heart mitochondria retain only a small degree of respiratory control, it must be concluded that occurrence of the oligomycin-sensitive ADP-ATP exchange activity correlates more directly with a high degree of respiratory control than with the capacity to phosphorylate as measured by the P:O ratio. It appears significant that freezing and thawing also cause appearance of ATPase activity. It can, therefore, be proposed that those factors leading to exposure or unmasking of ATPase activity are those causing loss of the oligomycin-sensitive component of the ADP-ATP exchange activity; conversely, occurrence of an oligomycin-sensitive ADP-ATP exchange may be characteristic of those systems in which the ATPase activity is latent or masked. Wadkins and Lehninger (15) have suggested a mechanism for the relationship between loss of acceptor control and loss of oligomycin-sensitivity of the ADP-ATP exchange reaction that involves a dissociation of the coupling enzyme system during the course of preparation of submitochondrial particles. This mechanism requires modification so that it can also account for increased ATPase activity; an obvious possibility is that the affinity for ADP decreases on loss of acceptor control, allowing an increase in ATPase activity. This point is under further investigation. Such a decrease in affinity for ADP has also been suggested by Zalkin et al. (31) on somewhat different grounds.

Of the various partial reactions of oxidative phosphorylation, the ADP-ATP and Pi-ATP exchange reactions have been somewhat controversial, and it has often been considered that one or the other may be catalyzed by enzymes not directly concerned in the mechanism of oxidative phosphorylation (49, 50). One type of evidence often cited against their relationship to oxidative phosphorylation is that certain submitochondrial preparations that are still capable of oxidative phosphorylation sometimes show little or no Pi-ATP (1, 8) or ADP-ATP (31) exchange activity. However, this argument may be fallacious, for if it is applied to other features of oxidative phosphorylation mechanisms, it can lead to absurd conclusions. For example, the dinitrophenol-stimulated ATPase activity of intact mitochondria has long been accepted as being catalyzed by an enzyme participating in oxidative phosphorylation. Yet in some submitochondrial sonic particles that are able to phosphorylate (cf. Reference 8, Table VI), dinitrophenol often produces no stimulation of ATPase activity beyond that given by Mg\(^{2+}\). Another case is the phenomenon of acceptor control. Intact mitochondria yield only low rates of electron transport in the absence of ADP, and considerable evidence has accumulated (49, 53, 54) that the stimulation of respiration given by ADP is due to its ability to act as a phosphate acceptor in oxidative phosphorylation. In many submitochondrial systems, however, there is little or no acceptor control of respiration, yet such systems may retain a large capacity for net oxidative phosphorylation. In fact, ADP may even produce reverse acceptor control in some submitochondrial systems; i.e. it may inhibit respiration and still serve as a phosphate acceptor in net oxidation phosphorylation (56, 57). It would be fallacious to conclude from such observations that dinitrophenol-stimulated ATPase activity or the phenomenon of respiratory control are not catalyzed by, or are the result of, the action of enzymes of oxidative phosphorylation. It appears equally unwarranted to conclude on the basis of existing evidence on submitochondrial systems that the oligomycin-sensitive ADP-ATP exchange reaction cannot be catalyzed by an enzyme participating in oxidative phosphorylation.

Since our data show that the oligomycin-sensitive ADP-ATP exchange reaction is specific for adenine nucleotides and is inhibited by atracyloside, it appears highly probable that the over-all exchange of externally added ADP and ATP as described in this paper includes the membrane translocation steps via the atracyloside-sensitive specific ADP-ATP "permease" of the mitochondrial membrane, postulated by Klingenberg et al. (43, 50). In terms of this hypothesis, the ADP-ATP exchange described in this paper may actually be taking place within the mitochondria, but requires action of the postulated membrane "permease" to account for the isotopic exchange observed in the external nucleotides. The relationship between the over-all
ADP ATP exchange and the ADP ATP transmembrane exchange is being investigated.

Further work is also being carried out on the appearance of soluble ATPase activity that accompanies the disappearance of oligomycin-sensitive ADP-ATP exchange activity in beef heart and liver mitochondria subjected to various treatments. An enzyme catalysing an ADP-ATP exchange reaction has also been separated from beef heart mitochondria in soluble form, purified considerably, and its characteristics have been examined. Reports on this work will be presented for publication elsewhere.

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Properties of an Oligomycin-sensitive Adenosine Diphosphate-Adenosine Triphosphate Exchange Reaction in Intact Beef Heart Mitochondria

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