The adenine nucleotide translocase was purified from bovine heart mitochondria and incorporated into membranes of phospholipid liposomes. The rate of transport of the adenine nucleotides was competitively inhibited by oleoyl coenzyme A with an approximate \( K_i \) of 1.0 \( \mu \)M. Significant inhibition was limited to those fatty acyl coenzyme A esters which are carnitine dependent for their oxidation in isolated mitochondria. Octanoyl coenzyme A was almost completely inactive as was palmitic acid and palmitoyl carnitine. By comparing the inhibitory characteristics of carboxyatractylate and bongkrekic acid with those of oleoyl-CoA, it was determined that the fatty acyl-CoA esters could produce inhibition whether the carrier was inserted into the liposome in either the conventional (65%) or reverse (30%) orientation. The results demonstrate that the interaction of long chain fatty acyl-CoA esters with the ADP/ATP carrier in a purified reconstituted system mimics their effects with isolated mitochondria and inverted submitochondrial particles. In general, these findings are consistent with the role of acyl-CoA esters acting as natural ligands and biological effectors of the translocase.

Long chain fatty acyl coenzyme A esters are potent reversible inhibitors of adenine nucleotide translocation in mitochondria (1), submitochondrial particles with inverted sidedness (2), and mitoplasts prepared by lubrol extraction (3). Because of their strikingly similar effects to those of the classical inhibitors atracetylate and bongkrekic acid, long chain fatty acyl-CoA esters have been proposed to function as natural ligands for the ADP/ATP carrier (4). However, not all investigators agree with this concept and some regard the acyl-CoA esters to be only nonspecific inhibitors of the translocase (5, 6). In view of the increasing amount of evidence which indicates that the adenine nucleotide translocase is the rate-limiting step for oxidative phosphorylation (6-8), it is very important to document the biochemical specificity of the acyl-CoA effect if the ligands are to be seriously considered as biological effectors of the carrier.

Analytical techniques for the isolation and purification of the ADP/ATP carrier from bovine heart mitochondria and its subsequent reconstitution in a liposome system have recently been developed (9-11). The use of these procedures offers a unique opportunity to establish rigorous criteria with which to test the specificity and sensitivity of the adenine nucleotide translocase to the fatty acyl-CoA esters. The present communication provides results which demonstrate that the reconstituted carrier is sensitive to the acyl-CoA in a specific manner. Furthermore, the interaction of the ligand with the receptor corresponds appropriately with the asymmetric orientation of the carrier in the liposome membrane.

**MATERIALS AND METHODS**

\([1^C]ADP\) (99.9% radiochemically pure) was purchased from New England Nuclear; hydroxyapatite and anion exchange resin AG 1-X8, 100-200 mesh, formate form, were purchased from Bio-Rad; carboxyatractylate was purchased from Boehringer Mannheim; bongkrekic acid was a gift from Professor W. Berends, University of Delft, The Netherlands; fatty acyl-CoA and carnitine esters were purchased from P-L Biochemicals, Inc.; phosphatidylcholine (egg, >99% pure), phosphatidylethanolamine (egg, >99% pure), and cardiolipin (beef heart) were purchased from Avanti Polar Lipids, Inc., Birmingham, AL.

Heavy beef heart mitochondria were prepared according to the procedure of Green et al. (12) and protein was determined by the method of Lowry et al. (13). The ADP/ATP carrier was purified according to published procedures (9-11) with slight modification. A mitochondrial pellet containing 50-60 mg of protein was dissolved in 2.5% Triton X-100, 100 mM Na\( \text{SO}_4 \), 10 mM Tricine-KOH, pH 7.4, and 1.0 mM EDTA at a concentration of 7 mg/ml. The suspension was incubated at 0 °C for 20 min and then centrifuged at 20,000 \( g \) for 10 min. A 1-ml aliquot of the supernatant was placed on a hydroxyapatite column (6 cm x 0.7 cm) that had been equilibrated with an elution buffer containing 0.5% Triton X-100, 10 mM Na\( \text{SO}_4 \), 10 mM Tricine-KOH, pH 7.4, and 0.1 mM EDTA, and the carrier protein which was eluted in the pass through fraction within 15 min was monitored by protein determinations.

The purified ADP/ATP carrier protein was incorporated into liposomes composed of phosphatidylethanolamine/phosphatidylcholine/cardiolipin in a ratio of 70:22:8 (9-11). A chloroform solution of the phospholipids was dried under nitrogen at room temperature and then vortex suspended in a medium containing 20 mM ATP, 100 mM Na\( \text{Cl} \), 0.5 mM Mg\( \text{Cl}_2 \), and 10 mM Tricine-KOH, pH 7.5, at a concentration of 33 mg/ml. The phospholipid suspension was sonicated at 40-50 watts using a Branson Sonifier with a microtip for 15 min in an ice bath and centrifuged at low speed for 10 min. Approximately 200-300 \( \mu \)g of carrier protein in 0.2-0.4 ml of Triton X-100 solution was added to the sonicated liposome suspension and incubated at 0 °C for 20 min. The mixture was then subjected to a second sonication of 15-20 s in an ice bath. External ATP was removed by passage of the reconstituted carrier through an anion exchange column (25 cm x 0.7 cm) (AG 1-X8, 100-200 mesh, formate form) that had been equilibrated with 136 mM glycerol solution (14). The fractions containing the proteoliposomes which were eluted with the glycerol buffer were pooled and used for the transport studies.

Adenine nucleotide transport was measured by a standard radioactive forward exchange assay (1). In a typical assay, 0.2-0.5 ml of the reconstituted carrier was added to a 1.0-ml incubation media containing 250 mM sucrose, 0.5 mM EDTA, and 20 mM 4-morpholinopropanesulfonic acid, pH 7.0, Vortex dispersed, and incubated for 4 min at room temperature. The reaction mixture was then incubated for an additional 2 min with or without the inhibitors and the forward exchange initiated by addition of 3.3 \( \mu \)Ci of \([1^C]ADP\) at a final concentration of 12297.
concentration of 10 μM. After incubation for 15 min at room tempera-
ture, the exchange was terminated by addition of 10 μM carboxy-
actylate and 10 μM bongkrekic acid and the external [14C]ADP
removed by passage of the reaction mixture through an anion ex-
change column (6 cm x 0.7 cm) (AG 1-X8, 100-200 mesh, formate
form) which was equilibrated with 136 mM glycerol. The proteolipo-
somes were eluted with 136 mM glycerol and 1.5-ml aliquots were
collected until the liposomes were quantitatively recovered. The
radioactivity was eluted with atractylate and 10%
removed by passage of the reaction mixture through an anion ex-
change column.

Sodium dodecyl sulfate gel electrophoresis was performed on 10%
carcinoid amide and 0.27% bisacrylamide gels using the discontinuous
buffer system of Neville (16) as previously described (17).

RESULTS

In order to prevent denaturation of the carrier protein following its extraction from the inner mitochondrial mem-
brane, it is characteristically preloaded with the tight binding
ligand, carboxyatractylate, which confers almost complete
protection during the purification (18). This procedure is not
applicable when the objective is to obtain a protein which
carries out active transport. Conditions have, therefore, been
modified and with the use of a very small hydroxyapatite
column, a more rapid purification has been accomplished
permitting a considerable amount of the unliganded carrier to
be actively reconstituted into the liposome system (11). The
carrier protein purified by hydroxyapatite chromatography as
described under “Materials and Methods” is a dimer of 60,000
daltons (18). Fig. 1 is a representative example of the purified
preparation used in these experiments which has been sub-
jected to sodium dodecyl sulfate gel electrophoresis. A distinct
protein band with the mobility equivalent to a molecular
weight of 30,000 is the subunit of the carrier (18). Using the
standard more lengthy purification procedure, an almost ho-
mundous protein (70-80% pure) can be obtained by chro-
matography on hydroxyapatite (17, 18).

The results in Table I demonstrate rates of adenosine nucleo-
tide transport in the reconstituted liposome system which are
comparable to values reported (9-11) and are almost com-
pletely inhibited by the combined addition of carboxyatract-
ylact and bongkrekic acid. The critical observation in this
series of experiments is the one showing that adenine nucleo-
tide translocation is also extremely sensitive to oleoyl-CoA.

The concentration curve for oleoyl-CoA inhibition of ade-
nine nucleotide transport in the liposome system shown in
Fig. 2 is almost identical with that obtained with either
mitochondria or submitochondrial particles (2). Furthermore,
the inhibition is competitive with ADP as it is in isolated
mitochondria (2, 3). The K_m for ADP of 14 μM obtained in
these experiments is similar to the value recently reported for
the reconstituted carrier system (11,19). A K_m for oleoyl-CoA
was calculated to be 1.0 μM. The value is similar to that
reported for rat liver mitochondria (3) but lower than the
figure given for palmitoyl-CoA inhibition of a reconstituted
carrier protein prepared from cholate-extracted bovine heart
mitochondria (20).

Fatty acyl-CoA inhibition of adenine nucleotide transloca-
tion in isolated mitochondria is limited to those thioesters
which are carnitine dependent for their subsequent oxidation
(2, 3). In fact, inhibition is released upon addition of carnitine
to the incubation medium which permits the transacylation
of the acyl-CoA to the acylcarnitine ester by the carnitine
palmitoyltransferase enzyme (2, 3). In the experiments shown
in Table II, all of the acyl-CoA esters tested except octanoyl-
CoA produced a strong inhibition of adenine nucleotide trans-
port, again illustrating the marked similarity of the responses
in isolated mitochondria and the reconstituted liposome sys-

<table>
<thead>
<tr>
<th>Additions</th>
<th>Bound [14C]ADP</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min/mg</td>
</tr>
<tr>
<td>None</td>
<td>685</td>
</tr>
<tr>
<td>Carboxyatractylate + bongkrekic acid</td>
<td>26</td>
</tr>
<tr>
<td>Oleoyl-CoA</td>
<td>41</td>
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</table>

![Fig. 1. Sodium dodecyl sulfate gel electrophoresis of the peak protein fraction obtained by hydroxyapatite chromatography. Experimental and analytical techniques are described under “Materials and Methods.” A, standard molecular weights. B, protein bands from peak fraction; 30,000 daltons represents the ADP/ATP carrier subunit.](image)

![Fig. 2. Effect of increasing concentrations of oleoyl-CoA on the rate of transport in the reconstituted ADP/ATP carrier. Inset, double reciprocal plot of oleoyl-CoA inhibition of the concentration-dependent rate of ADP uptake. The assay for transport was carried out as described under “Materials and Methods.” /V represents 1/ADP bound (nanomoles/min/mg of protein); O—O, control; △—△, oleoyl-CoA, 4 μM; □—□, oleoyl-CoA, 9 μM.](image)
Acyl-CoA Effect on the Reconstituted ADP/ATP Carrier

**TABLE II**

Effect of different fatty acyl-CoA esters, palmitic acid, and palmitoyl carnitine on the rate of ADP transport of the reconstituted ADP/ATP carrier

The transport assays were carried out as described under “Materials and Methods.” The amount of protein in the reconstituted carrier was 0.2 µg and the concentration of the added ligands was 10 µM.

<table>
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<tr>
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<tr>
<td>None</td>
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<td>Octanoyl-CoA</td>
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<tr>
<td>Palmitic acid</td>
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<tr>
<td>Palmitoyl carnitine</td>
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</table>

**TABLE III**

Relationship of the inhibitors of adenine nucleotide transport to the orientation of the reconstituted carrier in the liposome

The transport assays were carried out as described under “Materials and Methods.” The amount of protein in the reconstituted carrier was 0.2 µg.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Bound [%]ADP</th>
<th>Orientation of the carrier</th>
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<tbody>
<tr>
<td>None</td>
<td>700</td>
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<tr>
<td>Carboxyatractylate</td>
<td>5 µM</td>
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<td>Bongkrekic acid</td>
<td>10 µM</td>
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**DISCUSSION**

The present results provide evidence that the interaction between the ADP/ATP carrier and long chain fatty acyl-CoA esters in a purified reconstituted system is identical with that observed in isolated mitochondria and submitochondrial particles. An important finding brought out in previous studies (2, 23, 24) was that, unlike atracylactate and bongkrekic acid which bind and inhibit the carrier asymmetrically, long chain fatty acyl-CoA esters recognize receptor sites on both the cytosolic and matrix side of the inner mitochondrial membrane. Moreover, on the cytosolic side of the membrane, the acyl-CoA esters resemble atracylactate in their kinetic effects, while on the matrix side, they resemble bongkrekic acid (4). Whereas the present results might seem predictable, it was, nevertheless, reassuring to find that they confirm the fact that in a reconstituted system as in the natural environment, the acyl-CoA esters were still able to recognize either conformation of the receptor as dictated by the orientation of the carrier in the membrane.

The role of the ADP/ATP carrier in energy-linked respiration is an important factor in assessing the potential significance of the present study. Whereas there has been disagreement in the past, more recent evidence (6-8) favors the probability that the adenine nucleotide translocase is the rate-limiting step in oxidative phosphorylation. This condition implies that the carrier, like other rate-limiting enzymes, must be carefully regulated. It also seems reasonable to consider the possibility that the receptors on the carrier fortuitously recognize atracylactate and bongkrekic acid, and that the fatty acyl-CoA esters may represent the natural ligands and biological effectors of the translocator. The present study supports...
this concept and future efforts will be directed to providing
evidence for the existence of physiological or pathophysiological conditions under which the ADP/ATP carrier is regulated
and, in turn, affects energy-linked respiration.

Acknowledgment—We appreciate the helpful discussions of H. A. Lardy, Enzyme Institute and Department of Biochemistry, University of Wisconsin, Madison, WI.

REFERENCES