Use of an Antibody to Study the Location of Cardiolipin in Mitochondrial Membranes

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SUMMARY

Rabbit antiserum to cardiolipin, which is reactive with the polar head but not the nonpolar fatty acid moieties of cardiolipin, was used to explore the location of the polar head of cardiolipin in mitochondrial membranes. Only a few per cent of the cardiolipin in intact mitochondria from rat liver, blowfly flight muscle, Saccharomyces cerevisiae, and Neurospora and none of the cardiolipin in intact beef heart mitochondria is available for binding of anticardiolipin antibody. Freezing and thawing, aging at 45°, or sonication, in the absence or presence of the antibody, increased only slightly the anticardiolipin antibody binding activity of various types of mitochondria. The only mitochondrial preparation showing complete ability to bind anticardiolipin antibody was a mitochondrial precursor fraction isolated from glucose-repressed, anaerobic yeast cells. The isolated outer and inner membrane fractions from rat liver mitochondria also showed very little capacity to bind the antibody; both the cytoplasmic side and the matrix side of the inner membrane, which contains most of the cardiolipin showed little antibody binding activity. Removal of the F1 ATPase molecules from inner membrane vesicles of beef heart mitochondria also failed to unmask antibody binding activity. Neither oxidative phosphorylation nor energy-linked Ca++ transport in intact rat liver mitochondria also influenced by addition of excess anticardiolipin antibody. It is concluded that the polar heads of most of the cardiolipin molecules in the mitochondrial membranes are buried within the structure of the membrane or shielded by the binding of other membrane components.

The antigenic activity of cardiolipin (diphosphatidylglycerol) has been extensively studied in connection with its use in the serodiagnosis of syphilis. Anticardiolipin antibody has been found to combine with the polar head of the cardiolipin mole-

\[
\begin{align*}
\text{CH}_2 & \quad \text{CH} \quad \text{CH}_2 \\
O & \quad O \\
\text{O} = \text{C} & \quad \text{C} = \text{O} \\
R^1 & \quad R^2
\end{align*}
\]

The specific antigenic activity is associated with the bridging phosphate group between the glycerol residues and the free 2-hydroxyl group of the interior glycerol molecule (1-3). These findings suggested that the anticardiolipin antibody might be used as a specific probe to locate the antigenic "heads" of cardiolipin molecules in membranes containing this phospholipid and thus to explore aspects of the molecular topology of the membrane. In this paper we examine the binding of this antibody to mitochondrial membranes, which characteristically contain large amounts of cardiolipin.

EXPERIMENTAL PROCEDURE

Membrane Preparations—Rat liver mitochondria and mitochondrial fractions enriched in outer membrane, inner membrane plus matrix, and inner membrane minus matrix were prepared according to the method of Schnaitman and Greenawalt (4), sonic particles of rat liver mitochondria according to the method of Gregg (5), and mitochondria from Saccharomyces cerevisiae strain D 261 by the method of Guarnieri et al. (6). Membrane fractions enriched in cardiolipin were isolated from S. cerevisiae grown under anaerobic conditions as described by Goffeau et al.1 Neurospora crassa cells and mitochondria were generously supplied by David Beck, and flight muscle mitochondria from the blowfly Phormia regina by Dr. B. Sacktor. Beef heart mitochondria were prepared as described by Settlemire et al. (7) and beef heart mitochondrial S-particles and T2-particles according to Racker (8). Erythrocytes were obtained from rabbit or rat whole blood collected with 1 part of 3.8% sodium citrate per 9 parts blood.

Analytical Methods—Lipids were extracted and determined according to methods described by Fleischer et al. (9). Monoamine oxidase and cytochrome oxidase were used as marker enzymes to estimate the purity of the inner and outer mitochondrial membrane fractions (4). Lipids for the preparation of

the immunizing and standard antigen and for use as chromatographic markers were obtained from Supelco, Inc., Bellefonte, Pennsylvania. Protein was determined by the method of Murphy and Kies (10).

**Antiserum Preparation**—The preparation of the immunizing antigen solution, the treatment of the rabbits, and the collection of antisera were conducted exactly as described by Inoue and Nojima (2). White male rabbits, 3 to 5 kg, were obtained from the Bar-F Rabbitry, Perry Hall, Maryland. Eight rabbits were used, six for the production of antisera and two for control sera.

**Determination of Concentration of Anticardiolipin Antibody**—The serum from each rabbit was assayed for antibody activity using a modification of the “Quantitative Slide Test” described by the Venereal Disease Research Laboratory (referred to as VDRL) of the United States Public Health Service (11). The standard antigen solution used for this test contained 900 mg of cholesterol, 300 mg of phosphatidylcholine, and 30 mg of cardiolipin in 100 ml of ethanol. To carry out the assay, 20 μl of standard antigen suspension were mixed with 50 μl of antiserum. The amount of flocculation produced was graded visually on a 0, 1+, 2+, 3+, 4+ basis. At the end of the 3-week immunization period the average antiserum titer was 1:64, that is 50 μl of a 1 to 64 dilution of the antiserum was sufficient to induce 2+ flocculation in 20 μl of the standard antigen suspension.

Antiserum sterilized by passing through a 0.22 μm Millipore filter was stable at 5° for at least 2 months. Fresh, unfiltered antiserum was stable at -20° or -196° for at least 1 week. The activity of the antiserum was only slightly decreased by lyophilization and storage of the dry powder at -20° for 1 week.

**Measurement of Antibody Binding to Mitochondria and Other Membranes**—Graded amounts of the membrane preparation were suspended in 200 μl of 0.15 M sodium chloride and mixed with 500 μl of various dilutions of the antiserum. The mixture was shaken for 4 min at 25° and then centrifuged to sediment the membrane with its bound antibody. To determine the amount of free antibody remaining in the clear supernatant medium, a 50-μl aliquot of the latter was allowed to react with the standard antigen in the VDRL slide test, and the amount of flocculation recorded. If no flocculation occurred (recorded as 0), it was assumed that 100% of the antibody originally mixed with the membrane preparation was bound by the latter; if maximum flocculation (1+1) was observed, no antibody was bound by the membrane. Flocculation reactions of 1+, 2+, and 3+ thus correspond to binding of approximately 75%, 50%, and 25% of the added antibody (12).

Quantitative comparisons of the antibody binding activity of different membrane preparations were made as follows. Each antiserum was first diluted to such a concentration that 50 μl would produce maximum flocculation of 20 μl of the standard antigen suspension, which contained 0.4 nmole of cardiolipin. Each membrane preparation was then titrated with 500 μl of such a standardized dilution of antiserum (equivalent to 4.0 nmoles of cardiolipin) as well as with two additional, more concentrated solutions of known concentration prepared from the same antiserum. The amount of antibody bound by each membrane preparation was computed as the average value obtained from titrations with three different antiserum concentrations. For these calculations those flocculation reactions giving a score of either 0 or 4+ on the supernatant medium containing the remaining unbound antibody were not used. The complete details of a typical experiment, showing the procedures for scoring and calculating the amount of bound antibody, are given under "Results."

**RESULTS**

**Amount of Antibody Bound by Intact Rat Liver Mitochondria**—Typical experimental data and calculations used to construct a curve (Fig. 1) describing the binding of anticardiolipin antibody to intact rat liver mitochondria are given in Table I. Graded amounts of mitochondria (from 0.50 to 3.0 mg of protein) suspended in 200 μl of 0.15 M potassium chloride were added to 500 μl of three concentrations of standardized antiserum, representing 1:16, 1:12, and 1:8 dilutions, equivalent to 4, 6, and 8 nmols of cardiolipin, respectively. After a 4-min incubation at 25°, the mixture was centrifuged and the amount of unbound antibody remaining in the supernatant medium was estimated, using 50-μl aliquots of the latter in the VDRL slide test. The

![Graph](https://via.placeholder.com/150)

**Table 1**

<table>
<thead>
<tr>
<th>Mitochondria added</th>
<th>Flocculation test score on unbound antibody remaining in the medium with following antibody equivalents added</th>
<th>Added antibody bound by mitochondria with following antibody equivalents added</th>
<th>Antibody equivalents bound with following antibody equivalents added</th>
<th>Average of antibody equivalents bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg protein</td>
<td>8 6 4</td>
<td>8 6 4</td>
<td>8 6 4</td>
<td>8 6 4 Total</td>
</tr>
<tr>
<td>3.00</td>
<td>1 1 0</td>
<td>75 75 100</td>
<td>6 4.5</td>
<td>5.2 1.7</td>
</tr>
<tr>
<td>2.00</td>
<td>2 2 0</td>
<td>50 50 100</td>
<td>4 3</td>
<td>2.5 1.7</td>
</tr>
<tr>
<td>1.00</td>
<td>3 2 0</td>
<td>25 50 100</td>
<td>2 3</td>
<td>2.5 2.5</td>
</tr>
<tr>
<td>0.75</td>
<td>4 3 2</td>
<td>25 25</td>
<td>1.5 2</td>
<td>1.7 2.3</td>
</tr>
<tr>
<td>0.50</td>
<td>4 3 3</td>
<td>25 25</td>
<td>1.5 1.7</td>
<td>1.2 2.4</td>
</tr>
</tbody>
</table>

* Average antibody equivalents bound per mg of mitochondrial protein is 2.1 ± 0.3.
flocculation test score was then converted into the percentage of the added antibody that was bound by the mitochondria. The amounts of antibody bound from the three antisera dilutions were then averaged and the results expressed as antibody equivalents, defined as the amount of antibody required to titrate 1.0 nmole of cardiolipin in the form of the standard antigen. As is seen in Table I, intact rat liver mitochondria bind 2.1 antibody equivalents per mg of mitochondrial protein.

Fig. 1 shows a plot of the number of antibody equivalents bound per mg of protein as a function of mitochondrial concentration, from the data of Table I. It is seen that intact rat liver mitochondria bind the antibody in an approximately linear manner over the range 0.50 to 3.0 mg of mitochondrial protein. At higher concentrations of mitochondria less antibody is bound per mg of protein than in the linear zone, presumably because some of the antibody binding sites are masked through mitochondrial aggregation. At lower concentrations of mitochondria, below the linear zone, somewhat more antibody is bound per mg of protein than in the linear zone; presumably the enhanced swelling of mitochondria at low concentrations (13) causes exposure of more antibody binding sites.

The antibody binding capacity of fresh intact rat liver mitochondria varied only slightly among different preparations with high respiratory control ratios as is seen in Table II. However, mitochondria with low respiratory control ratios often exhibited much greater antibody binding capacity compared to tightly coupled mitochondria. On the other hand, cycles of freezing and thawing had no effect on the ability of mitochondria to bind antibody.

From the number of antibody equivalents bound by the mitochondria it is possible to estimate the fraction of all cardiolipin molecules in the intact mitochondria which are available for reaction with the antibody. Data in Table I show that intact rat liver mitochondria bind, per mg of total protein, an amount of antibody capable of reacting with 2.1 nmole of cardiolipin. Because intact rat liver mitochondria contain 27 nmole of cardiolipin per mg of protein, as determined following total lipid extraction and thin layer chromatography, it may be concluded that less than 9% of the cardiolipin in intact rat liver, yeast, and blowfly mitochondria and none of the cardiolipin molecules of heart mitochondria are accessible to the antibody.

Neither intact erythrocytes nor Neurospora cells bound antibody (Table III). However, the microsome fraction of rat liver bound a small amount of antibody; the significance of this effect will be considered below.

**Table II**

Antibody binding to rat liver mitochondrial preparations

Values for antibody binding are expressed as an average ± standard deviations. A total of five binding determinations was made on each preparation.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Respiratory control ratio</th>
<th>Antibody equivalents bound per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact mitochondria ..........</td>
<td>4.8</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Intact mitochondria ..........</td>
<td>4.5</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Intact mitochondria ..........</td>
<td>4.5</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Frozen-thawed mitochondria</td>
<td>0</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

**Table III**

Binding of anticardiolipin antibody to mitochondria from other species and to other membrane systems

The numbers in parentheses indicate the number of preparations tested. The blowfly mitochondria had been frozen and thawed before the experiment.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Range tested</th>
<th>Antibody equivalents bound per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospora mitochondria</td>
<td>0.5-3.0 mg protein</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>Yeast mitochondria (5)</td>
<td>0.5-3.0 mg protein</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>Blowfly mitochondria (4)</td>
<td>0.5-6.0 mg protein</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Beef heart mitochondria</td>
<td>0.5-10 mg protein</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
<td>1.0-8.0 mg protein</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Intact erythrocytes (rat)</td>
<td>0.5-2.0 mg, wet weight</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Intact erythrocytes (rabbit)</td>
<td>2.0 mg, wet weight</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Intact Neurospora crassa</td>
<td>10-40 mg, wet weight</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

**Table IV**

Antibody binding capacity of lipid-extracted rat liver membranes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Range tested</th>
<th>Antibody equivalents bound per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracted with chloroform-methanol</td>
<td>0.5-10 mg protein</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1-20 mg protein</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracted with ethanol</td>
<td>0.5-2 mg protein</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.5-2 mg protein</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The presence of significant antibody binding activity in the microsomal lipids, together with other observations, led us to examine the specificity of the anticardiolipin antibody with purified phospholipids and other compounds. Although the results are to be described elsewhere, the most pertinent

* M. Guarnieri, D. J. Eisner, and A. L. Lehninger, unpublished results.
finding was that only phosphatidylinositol of all the lipids tested showed significant capacity to bind anticardiolipin antibody. It yielded about 25 to 50% of the activity shown by cardiolipin. This observation suggests that the significant reactivity of the lipids from rat liver microsomes with the antibody is actually due to their high phosphatidylinositol content, about 100 nmoles per mg of protein (14), an amount capable of binding 5 to 10 times the antibody added in the flocculation tests. Thus it appears that the phosphatidylinositol of microsomal membranes is also relatively inaccessible to antibody.

Factors Affecting Accessibility of Mitochondrial Cardiolipin to Antibody—The data collected in Table VI indicates how accessibility to the antibody is affected by aging of freshly prepared, intact rat liver mitochondria. For comparison, results from antibody binding studies with yeast and blowfly muscle mitochondria are shown. When rat liver mitochondria were aged at 45° for 15 to 30 min in the presence of antibody, the amount of antibody bound was approximately doubled. Similar results were obtained by brief (30 sec to 2 min) sonication of rat liver mitochondria in the presence of antibody. However, if antibody was added after liver mitochondria were aged or sonicated, no increase in antibody binding occurred, indicating that the cardiolipin is not exposed in the vesicles formed from the inner mitochondrial membrane (15), which contains 90% of the total cardiolipin.

Although beef heart mitochondria are very rich in cardiolipin they failed to bind antibody even after aging or sonication in the presence of antibody. It is of significance, however, that the cardiolipin of a cytoplasmic membrane fraction of anaerobically grown yeast cells containing early precursors of mitochondria was almost totally accessible to the antibody.

### Table V

**Antibody binding activity of lipid extracts from various membranes**

Ethanol solutions of the membrane extracts or the purified lipids were added to an ethanol solution of phosphatidylcholine and cholesterol such that 1 ml of the resulting solution contained 9.0 mg of cholesterol, 3.0 mg of phosphatidylcholine, and 500 times (nanomoles of Pi) the various extracts or the purified lipids listed below. One milliliter of ethanol solution containing the test lipid plus phosphatidylcholine and cholesterol was mixed with 9 ml of buffered NaCl solution to yield a VDRL antigen solution, of which 20 μl were mixed with 50 μl of a standardized antiserum solution in the VDRL microflocculation assay.

<table>
<thead>
<tr>
<th>Lipid source</th>
<th>Amount of phospholipid added to 20 μl of standard antigen</th>
<th>Flocculation reaction with standard antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles total phospholipid phosphorus</td>
<td>nmoles cardiolipin</td>
</tr>
<tr>
<td>A. Unfractionated extracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver mitochondria</td>
<td>2.50</td>
<td>0.40</td>
</tr>
<tr>
<td>Rat liver mitochondria</td>
<td>1.25</td>
<td>0.20</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
<td>4.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Yeast mitochondria</td>
<td>4.00</td>
<td>1.48</td>
</tr>
<tr>
<td>Beef heart mitochondria</td>
<td>2.00</td>
<td>0.40</td>
</tr>
<tr>
<td>B. Purified phospholipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.40</td>
<td>0.00</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>0.70</td>
<td>0.00</td>
</tr>
</tbody>
</table>

### Table VI

**Accessibility of mitochondrial cardiolipin to antibody**

In the tests for antibody binding to aged mitochondria, mitochondria were incubated in antibody solution for the time and temperature described.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Antibody equivalents bound (nmoles/mg protein)</th>
<th>Cardiolipin accessible to antibody (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh (4)</td>
<td>2.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Aged 45°, 15 min (4)</td>
<td>3.7</td>
<td>13.7</td>
</tr>
</tbody>
</table>

### Table VII

**Phospholipid composition of rat liver mitochondrial membranes**

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Phosphatidic acid</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidylethanolamine</th>
<th>Phosphatidylinositol</th>
<th>Cardiolipin % total lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>170</td>
<td>48</td>
<td>28</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Inner membrane</td>
<td>130</td>
<td>42</td>
<td>32</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Lubrol pellet (inner membrane)</td>
<td>320</td>
<td>45</td>
<td>32</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Outer membrane</td>
<td>360</td>
<td>44</td>
<td>28</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Sonic particles</td>
<td>500</td>
<td>42</td>
<td>38</td>
<td>4</td>
<td>17</td>
</tr>
</tbody>
</table>

**Intramitochondrial Location of Cardiolipin**—Because aging, a process which promotes swelling and rupture of the outer membrane, enhanced the accessibility of the cardiolipin of yeast, blowfly, and liver mitochondria to the antibody, mitochondrial subfractions were prepared to determine which mitochondrial membrane had the greatest capacity to bind antibody. For reference, the phospholipid composition of the inner and outer mitochondrial membranes of rat liver mitochondria is shown in Table VII; it is seen that cardiolipin makes up about 7% of the outer membrane lipids and about 20% of the inner membrane lipids. Because the outer membrane contains about 10% and the inner membrane about 90% of the total mitochondrial membrane protein (4), there is about 10 times as much cardiolipin in the inner membrane as in the outer. The amounts of antibody bound by the outer membrane fraction and the inner membrane fraction are shown in Table VIII. The outer membrane fraction binds on the average over twice as much antibody as an equiva-
l)rotein, Table IX. Recause this inner membrane fraction con-
with Lubrol WX, the resulting inner membrane fraction showed
preparations bind no more antibody than untreated preparations.
brane fraction. Frozen and thawed or sonicated inner membrane
inner membrane marker enzyme, is present in the outer mem-
finding that about 10% of the total cytochrome oxidase, an
account for the enhanced antibody binding. However, micro-
almost all of the mitochondrial phosphatidylinositol, equivalent
to 70 nmoles per mg of outer membrane protein, which may
account for the enhanced antibody binding. However, micro-
osmes, which contain about 100 nmoles of phosphatidylinositol
per mg of protein, have very low antibody binding capacity. It
is obvious that antibody binding by the outer membrane prepara-
tion cannot be solely accounted for by contamination with inner
membrane material, whose magnitude is indicated by the
finding that about 10% of the total cytochrome oxidase, an
inner membrane marker enzyme, is present in the outer mem-
brane fraction. Frozen and thawed or sonicated inner membrane
preparations bind no more antibody than untreated preparations.
When the intact inner membrane-matrix fraction, which con-
tains about 25 nmoles of cardiolipin per mg of protein, is treated
with Lubrol WX, the resulting inner membrane fraction showed
a 4-fold increase in the amount of antibody bound per mg of
protein, Table IX. Because this inner membrane fraction con-
tains 73 nmoles of cardiolipin per mg of protein (cf. Table VII),
the total antibody-accessible cardiolipin in inner membrane
preparations is still only 64.0% compared to 9% for the inner
membrane-matrix fraction. Similarly, only 11% of the total
cardiolipin of the sonic particles was accessible to antibody.
Since the outer vesicular surface of Lubrol-treated or sonic-
treated inner membrane preparations correspond to the matrix
side (M side) of the inner membrane, it is clear that the matrix
surface of the inner mitochondrial membrane is only slightly
more reactive with antcardiolipin antibody than the cytoplasmic
surface. When intact inner membrane preparations were mixed
with antibody solutions and incubated at 45°C for 15 min, the
antibody-reactive sites increased about 3- to 5-fold, equivalent
to about 30% accessibility of the total cardiolipin. It would
therefore appear from these experiments that the polar heads
of the cardiolipin molecules are largely inaccessible from either
the matrix or cytoplasmic surface of the inner membrane.

Table IX

Binding of antibody by submitochondrial vesicles

The preparations of intact membrane plus matrix were incubated at 45°C for 15 min in the presence of antcardiolipin antiserum.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Antibody equivalents bound per mg of protein</th>
<th>Cardiolipin accessible to antibody</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact mitochondria (4)</td>
<td>2.6 ± 0.3</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>Inner membrane plus matrix (4)</td>
<td>3.0 ± 0.2</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>Outer membrane (6)</td>
<td>5.6 ± 0.3</td>
<td>22.0</td>
<td></td>
</tr>
</tbody>
</table>

a The numbers in parentheses indicate the number of preparations tested.

Table X

Antibody binding to particles deficient in ATPase activity

The preparations tested most of the membrane cardiolipin is antibody-inaccessible, and that the membrane components responsible for this inaccessibility are extremely stable. The only native mitochondrial fraction in which the cardiolipin was found to be accessible to antcardiolipin antibody is a mitochondrial precursor fraction isolated from anaerobically grown yeast cells. This fraction, which lacks cytochromes, contains oligomycin-sensitive ATPase activity. This fact suggests that the ATPase enzyme complex is not one of the membrane components that masks or covers the polar head of cardiolipin. However, because of the importance of the ATPase enzyme complex to mitochondrial function, the relation between ATPase activity and antcardiolipin antibody binding was further studied. The ability of inner membrane vesicles stripped of oligomycin-sensitive ATPase activity to bind antibody was examined.

The results of this experiment are shown in Table X. Beef heart S-particles prepared by sonicating beef heart mitochondria and TU-particles prepared by treating S-particles with trypsin and urea (8) were found to have negligible capacity to bind the antibody. The very slightly increased ability of the TU-particles to bind the antibody is not in proportion to the 95% loss in the ATPase activity of these particles. Thus in beef heart mitochondria it does not appear likely that the polar heads of the membrane cardiolipin serve as binding sites for the oligomycin-sensitive ATPase enzyme complex.

Reaction of Antibody with Delipidized Mitochondrial Enzymes and Other Proteins—In order to determine whether or not membrane proteins in general have the capacity to cover the antigenic head of the cardiolipin molecule, several lipid-free mitochondrial proteins were used to prepare cardiolipin-protein complexes, which were then allowed to react with the antibody. The delipidized proteins, suspended in 0.15 M NaCl, were mixed with cardiolipin in the same fashion used to prepare the immunizing antigen complex. When delipidized cytochrome c and a partially purified preparation of 3-hydroxybutyrate dehydrogenase (16) were mixed with cardiolipin, they inhibited antibody binding by the lipid to approximately 25 and 50%, respectively. When the total delipidized membrane proteins of rat liver mitochondria, liver microsomes, and rat erythrocytes were mixed with pure cardiolipin more than 75% of the added lipid became antibody-inaccessible. However, the degree of inhibition of antigenic activity varied somewhat with the technique used to

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extract the membrane lipids. Delipidized yeast mitochondria inhibited only slightly (less than 12%) the binding of antibody to the cardiolipin antigen.

**Effect of Ca**+ on Antibody Accessibility**—**Addition of CaCl2 (80 nmoles per mg of protein) significantly decreased the already small degree of binding of antcardiolipin antibody to intact rat liver mitochondria. The effect was not observed with similar concentrations of Mg2+, Mn2+, Sr2+ or with 8 nmoles of La3+ per mg of protein. Dinitrophenol partially reversed this inhibition. Control tests showed that Ca2+ did not interfere with the interaction between the standard antigen and the antibody. Treatment of mitochondrial sonic particles or Lubrol membranes with EDTA had no effect on antibody binding.

**Effect of Anticardiolipin Antibody on Mitochondrial Function**—There was no effect on the State 4-State 3 respiratory transitions, nor in the stimulation of respiration by Ca++, as measured with the oxygen electrode, of rat liver mitochondria which had been treated (20) and antibody binding studies (21) on erythrocyte membranes and on myelin. However, no more than 30% of the total membrane cardiolipin becomes accessible to antibody following such treatments. Complete reactivity of the mitochondrial cardiolipin with antibody was observed only by extracting the cardiolipin from the mitochondria with chloroform-methanol mixtures.

Beef heart mitochondria, which contain about four times as much cardiolipin as rat liver mitochondria, failed to bind any detectable amount of antcardiolipin antibody, even after they were aged at 45° for 30 min. Moreover, only a very small amount of antibody was bound by beef heart sonic particles. The complete failure of intact beef heart mitochondria to bind antcardiolipin antibody suggested the possibility that the small amounts of antibody bound by intact liver mitochondria may be due to the presence in the latter of reactive antigens other than cardiolipin.

We have found that phosphatidylinositol but no other phospholipid reacts significantly with antcardiolipin antibody. Since beef heart mitochondrial lipids contain only 2 to 3% of phosphatidylinositol (14), whereas those from rat liver mitochondria contain up to 10% phosphatidylinositol, all of which is concentrated in the outer membrane (Table VII), it appears possible that none of the cardiolipin in intact rat liver mitochondria is accessible to the antibody and that the limited antibody binding observed in intact rat liver mitochondria is due to outer membrane phosphatidylinositol. Our observations also suggest that cardiolipin is probably not involved in the binding of F1 ATPase molecules to the inner mitochondrial membrane. It may be noted, however, that acidic phospholipids appear to be essential components of the electron transfer process (17). Moreover, a portion of the cardiolipin of beef heart mitochondria is tightly bound to cytochrome oxidase (18) and can be isolated from a proteolipid fraction (19).

Our results also suggest that phosphatidylinositol is located in the microsomal membrane such that its polar head is obscured by other membrane components. Thus two types of acidic phospholipids seem to be “buried” in their membranes, but this architecture is not characteristic of all membrane lipids. Phospholipase D readily hydrolyzes phosphatidylethanolamine of intact mitochondria, indicating that the polar head of this phospholipid is largely exposed. Moreover, the polar heads of some lipids are exposed in other membranes, as is shown by phospholipase C treatment (20) and antibody binding studies (21) on erythrocyte membranes and on myelin (22).

There are some indications that cardiolipin must be present in a specific three-dimensional micellar arrangement in order to be immunogenic and to be reactive with the antcardiolipin antibody. For example, Inoue and Nojima (2) and Kataoka and Nojima (23) have shown that phosphatidylethanolamine is a necessary auxiliary lipid for the immunogenicity of cardiolipin. Moreover, Azzi et al. (24) have shown that phosphatidylethanolamine influences the binding of cardiolipin to cytochrome c. Thus cardiolipin must be oriented in the membrane in such a way that the phospholipid-phosphatidylinositol interaction may occur.

The experimental approach described in this paper appears to have some general applicability as a means of probing the molecular topology of other types of membranes. Such studies recently have been reported for erythrocyte membranes by Nanni et al. (21) and for myelin membranes by Rapport (22). Other antiphospholipid antibodies have been reported, such as antiphosphatidylcholine (25), and antiphosphatidylglycerol (26), and antiphosphatidylserine (27). Antibodies to several sphingolipids of neural tissues (27) have also been reported; they promise to be useful probes in topochemical studies of the membranes of the nervous system.

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Use of an Antibody to Study the Location of Cardiolipin in Mitochondrial Membranes
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