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 percent 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 were 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.

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. 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 T-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 serodiagnosis of syphilis. Anticardiolipin antibody has been found to combine with the polar head of the cardiolipin mole-

The antigenic activity of cardiolipin (diposphatidylglycerol) has been extensively studied in connection with its use in the

CH₂ CH₂ CH₂
O O
\[O=C (C\rightarrow O)\]
R₁ R₂
Antigenic portion

\[\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{CH₂ CH₂} & \quad \text{O} \quad \text{O} \\
\text{R₁} & \quad \text{R₂}
\end{align*}\]

1 A. Goffeau, W. X. Balcavage, J. W. Greenawalt, J. R. Mattoon, and A. L. Lehninger, manuscript in preparation.
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 phosphatidylethanolamine, 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 was 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 (11) 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 just 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 nmole 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 nmole 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

![Figure 1](http://www.jbc.org/)

**TABLE I**

| Mitochondria added | Flocculation test score on unbound antibody remaining in medium with following antibody equivalents added | Added antibody bound by mitochondria with following antibody equivalents added | Antibody equivalents bound with following antibody equivalents added | Average of antibody equivalents bound |
|---------------------|-------------------------------------------------|---------------------------------|-------------------------------------------------|---------------------------------|
| 3.00 mg protein     | 8 6 4                                           | 8 6 4                           | 8 6 4                                           | 8 6 4                           |
| 2.00 mg protein     | 8 6 4                                           | 8 6 4                           | 8 6 4                                           | 8 6 4                           |
| 1.00 mg protein     | 8 6 4                                           | 8 6 4                           | 8 6 4                                           | 8 6 4                           |
| 0.75 mg protein     | 8 6 4                                           | 8 6 4                           | 8 6 4                                           | 8 6 4                           |
| 0.50 mg protein     | 8 6 4                                           | 8 6 4                           | 8 6 4                                           | 8 6 4                           |

* Average antibody equivalents bound per mg of mitochondrial protein is 2.1 ± 0.3.
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 nmoles of cardiolipin. Because intact rat liver mitochondria contain 27 nmoles 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 microscopic fraction of rat liver bound a small amount of antibody; the significance of this effect will be considered below.

**Reaction of Mitochondrial Lipids with Antibody** The data in Table IV show that when the lipids are removed from rat liver mitochondria or microsomes by extraction with solvents, the remaining preparations no longer bind antibody. However, as shown in Table V, antibody-reactive material appears in the lipid extracts. In these experiments total lipid extracts of yeast, beef heart, and rat liver mitochondria, and of liver microsomes were mixed with the adjuvants phosphatidylcholine and cholesterol to yield antigen solutions in ethanol comparable in composition to the standard cardiolipin antigen solution. The amount of flocculation produced on mixing anticalciolin antibody with antigen suspensions prepared with such lipid extracts of membranes showed that the cardiolipin present in total lipid extracts of various mitochondria (determined chromatographically) was as reactive, mole for mole, as the standard antigen prepared from purified cardiolipin. Thus it is clear that the cardiolipin of mitochondria could be extracted in antibody-reactive form and that the other membrane lipids did not interfere in titrations of cardiolipin with antibody.

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

| Table IV |
| --- |
| **Antibody binding capacity of lipid-extracted rat liver membranes** |
| Range tested | Antibody equivalents bound per mg of protein |
| --- | --- |
| **Extracted with chloroform-methanol** |
| Mitochondria | 0.5–10 |
| Microsomes | 1–20 |
| **Extracted with ethanol** |
| Mitochondria | 0.5–2 |
| Microsomes | 0.5–2 |

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2 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 anticaldriolipin 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 microsomes. 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**

| Lipid source | Amount of phospholipid added to 20 μl of standard antigen | Flocculation reaction with standardized antiserum |
|--------------|----------------------------------------------------------|-----------------------------------------------|
|              | nmoles total phospholipid phosphorus | nmoles cardiolipin |
| A. Unfractionated extracts | | |
| Rat liver mitochondria | 2.50 | 0.40 | 4+ |
| Rat liver mitochondria | 1.25 | 0.20 | 2+ |
| Rat liver microsomes | 4.00 | 0.00 | 1+ |
| Rat erythrocytes | 4.00 | 0.00 | 0 |
| Yeast mitochondria | 4.00 | 0.48 | 4+ |
| Beef heart mitochondria | 2.00 | 0.40 | 4+ |
| B. Purified phospholipids | | |
| Phosphatidic acid | 0.40 | 0.00 | 0 |
| Cardiolipin | 0.40 | 0.40 | 4+ |
| Phosphatidylinositol | 0.70 | 0.00 | 1+ |

### 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.

| Preparation | Antibody equivalents bound | Cardiolipin accessible to antibody |
|-------------|---------------------------|-----------------------------------|
| Rat liver mitochondria | | |
| Fresh (4) | 2.0 | 7.5 |
| Aged 45°, 10 min (4) | 3.7 | 13.7 |
| Yeast mitochondria | | |
| Fresh (4) | 3.3 | 8.0 |
| Aged 45°, 30 min (4) | 5.8 | 14.0 |
| Beef heart mitochondria | | |
| Fresh (10) | <0.1 | 0 |
| Aged 45°, 30 min (12) | <0.1 | 0 |
| Blowfly mitochondria | | |
| Fresh (4) | 2.4 | 9.6 |
| Aged 45°, 15 min (4) | 4.5 | 18.0 |
| Mitochondrial precursor fraction from anaerobic, glucose-repressed yeast | | |
| Fresh (4) | 3.1 | 95 |

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

1 Frozen and thawed prior to testing.

### Table VII

**Phospholipid composition of rat liver mitochondrial membranes**

| Membrane | Phospholipid phosphorus | Phosphatidylcholine | Phosphatidyethanolamine | Phosphatidylserine | Cardiolipin |
|----------|-------------------------|---------------------|------------------------|-------------------|------------|
| Mitochondria | | | | | |
| Inner membrane | 170 | 48 | 28 | 7 | 16 |
| Inner membrane matrix | 130 | 42 | 32 | 0 | 26 |
| Lubrol pellet (inner membrane) | 320 | 45 | 32 | 0 | 23 |
| Outer membrane | 300 | 44 | 28 | 20 | 7 |
| Sonic particles | 500 | 42 | 38 | 1 | 17 |

**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-
TABLE VIII

| Preparation                     | Antibody equivalents bound per mg of protein | Total cardiolipin accessible to antibody |
|---------------------------------|---------------------------------------------|-----------------------------------------|
| Intact mitochondria (4)         | 2.6 ± 0.3                                   | 9.7                                     |
| Inner membrane plus matrix (4)  | 3.0 ± 0.2                                   | 9.0                                     |
| Outer membrane (6)              | 5.6 ± 0.3                                   | 22.0                                    |

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

TABLE IX

Binding of antibody by submitochondrial vesicles

The preparations of intact membrane plus matrix were incubated at 45° for 15 min in the presence of anticoagulant antibody.

| Preparation                        | Antibody equivalents bound per mg of protein | Cardiolipin accessible to antibody |
|------------------------------------|---------------------------------------------|----------------------------------|
| Inner membrane plus matrix (5)     | 3.0 ± 0.2                                   | 9.0                               |
| Same after aging (5)               | 10.3                                        | 30.5                              |
| Lubrol membranes (6)               | 12.1                                        | 16.4                              |
| Sonic particles (5)                | 10.9                                        | 11.0                              |

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

TABLE X

Antibody binding to particles deficient in ATPase activity

| Preparation                        | Antibody equivalents bound per mg of protein | ATPase activity |
|------------------------------------|---------------------------------------------|-----------------|
|                                    |                                             | nmoles F,,/10 min/mg |
| Beef heart mitochondria (4)        | <0.1                                        | 2.08            |
| Beef heart S-particles (4)         | <0.1                                        | 8.00            |
| Beef heart TU-particles (4)        | 0.62                                        | 0.16            |

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

Lent weight of intact mitochondria. This membrane contains 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, microsomes, 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 preparation cannot be solely accounted for by contamination with inner membrane material, whose magnitude is indicated by the finding that about 10% of the total cardiolipin oxidase, an inner membrane marker enzyme, is present in the outer membrane fraction. Frozen and thawed or sonicated inner membrane preparations bind no more antibody than untreated preparations.

When the intact inner membrane-matrix fraction, which contains about 35 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 contains 73 nmoles of cardiolipin per mg of protein (cf. Table VII), the total antibody-accessible cardiolipin in inner membrane preparations is still only 6.4% 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 sonicated 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 anticoagulant antibody than the cytoplasmic surface. When intact inner membrane preparations were mixed with antibody solutions and incubated at 45° 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.

**Reaction of Antibody with Membrane Preparations Stripped of F,, ATPase**

The preceding experiments show that in all mitochondrial membrane 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 anticoagulant antibody is a mitochondrial precursor fraction isolated from anaerobically grown yeast cells. This fraction, which lacks cytochrome c, 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 anticoagulant 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 partially purified preparation of β-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...
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 CaCl_2 (80 nmoles per mg of protein) significantly decreased the already small degree of binding of anticardiolipin antibody to intact rat liver mitochondria. The effect was not observed with similar concentrations of Mg_2+, Mn_2+, Sr_2+ or with 8 nmoles of La_3+ per mg of protein. Dinitrophenol partially reversed this inhibition. Control tests showed that Ca_2+ 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_2+, as measured with the oxygen electrode, of rat liver mitochondria which had been suspended in 0.25 M sucrose solution containing antibody equivalent to 16 to 32 nmoles of cardiolipin per mg of mitochondrial protein for 3 to 4 hours at 0-5°C. These observations indicate that neither oxidative phosphorylation nor energy-linked Ca_2+ transport require or involve cardiolipin molecules in a role which their polar heads are exposed in such a manner that they are accessible to the antibody. Moreover they also indicate that whatever membrane components are bound to the polar heads of the membrane cardiolipin, they are very stable and not displaced by the antibody.

DISCUSSION

The data reported in this paper indicate that intact mitochondria from rat liver, beef heart, yeast, and blowfly muscle show little or no capacity to bind rabbit antibody to cardiolipin, a characteristic component of mitochondrial lipids. Thus very little of the cardiolipin present in these membranes is oriented in such a way that the polar head of cardiolipin, which is the antigenic portion, is accessible to the antibody. The small amount of antibody binding that takes place with intact rat liver, yeast, and blowfly mitochondria can be increased only by heating or sonicating the mitochondria in the presence of the antibody. 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 anticardiolipin antibody, even after they were aged at 45°C 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 anticardiolipin 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 anticardiolipin 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 F_1 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 phosphatidylcholine 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 immunoactive and to be reactive with the anticardiolipin antibody. For example, Inoue and Nojima (2) and Kataoka and Nojima (23) have shown that phosphatidylcholine is a necessary auxiliary lipid for the immunogenicity of cardiolipin. Moreover, Atzi et al. (24) have shown that phosphatidylcholine influences the binding of cardiolipin to cytochrome c. Thus cardiolipin must be oriented in the membrane in such a way that cardiolipin-phosphatidylcholine 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 anti-lipid antibodies have been reported, such as antiphosphatidylinositol (23), antiphosphatidylcholine (25), and antidyrophosphoglycerol (26). 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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