Erythrocyte Membrane Lipids and Rh Antigen Activity*

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SUMMARY

The Rh antigens have never been totally and reproducibly obtained in free solution. Indirect evidence, based among other observations on the reversible loss of Rh antigen activity of lyophilized Rh-positive erythrocyte membranes with mercurial sulfhydryl reagents, suggests that the activity may depend on membrane protein. With the hypothesis that this membrane protein may be associated perhaps structurally with membrane lipid, studies were undertaken on the effects of organic solvents on lyophilized membrane Rh antigen activity. The antigen activity was abolished after extensive extraction with 100% I-butanol, but could be regenerated to approximately 50% of the unextracted membranes by the addition of certain lipids.

The present investigations establish that phospholipids are the only class of lipids which result in such regeneration. The binding of 14C-labeled lecithin of biological origin was measured in benzene solution, in sonicated aqueous dispersions, and in 5% methanol-ether dispersions. Regeneration of Rh activity was also followed, which showed that the binding of aqueous sonicated phospholipids (liposomes) was associated with the best regeneration and the maximum labeled lecithin bound.

The binding of 14C-fatty acids also occurred under similar conditions, but no associated regeneration of Rh antigen activity was observed. Similarly, an extensive series of nonphosphorus-containing lipids failed to regenerate the antigen activity.

Synthetic dipalmitoyl lecithin was found to bind to the 1-butanol-extracted membranes, but not to result in regeneration activity. Labeled phosphorylcholine showed little binding to these membranes, and large amounts of unlabeled phosphorylcholine only mildly depressed the binding of labeled lecithin. This suggests that the binding of the lecithin is predominantly hydrophobic.

A graded effect of different phospholipids was noted in regard to the extent of antigen regeneration.

Thus, the Rh antigen activity is dependent on the presence of bound phospholipid, containing at least one unsaturated fatty acid, with neither the polar nor the nonpolar portion of the molecule alone satisfying this requirement.

The lability of the Rh antigens led early workers to conclude that the chemical basis for Rh antigenicity differed from that of the ABO and MN systems (1, 2). Attempts to completely solubilize membrane Rh antigens (3, 4), or to obtain the antigens in soluble form from tissue fluids (6), have not been successful thus far. More recent investigations on the effect of sulfhydryl reagents on insoluble erythrocyte membrane Rh antigen activity have given indirect evidence suggesting the protein nature of these antigens. Using iodoacetamide (6) and both organic and inorganic mercurial sulfhydryl reagents (7), it was found that the membrane Rh activity was lost, but the activity could be regenerated, in the case of the mercurials, after incubation of the preparation with an excess of thiol (7). This reversibility tended to exclude some type of nonspecific denaturation reaction and supported the hypothesis that free sulfhydryl groups were required in that portion of the erythrocyte membrane protein responsible for the Rh antigen activity. Quantitative data revealed, however, that the share of the total membrane protein sulfhydryl groups apparently having to do with the Rh antigen activity was small (8).

The possibility has been considered that the Rh antigen related membrane protein might be bound to membrane lipid in a structurally important way. A number of investigators have succeeded in solubilizing red cell membranes with both organic reagents, such as 90% I-butanol, and detergents, but these procedures resulted in products having no demonstrable Rh antigen activity by hemagglutination inhibition (9-11). This hypothesis was further tested by a series of experiments in which the lyophilized erythrocyte membranes were extracted with water-free organic solvents and the Rh membrane activity measured after removal of these reagents. In contrast to the situation with the lower alcohols, where the Rh antigen activity was lost in all studies, this activity was preserved after only a few drops of 1-butanol were added and then removed, but was lost after a large amount of solvent was added and separated from the residue by filtration. This observation pointed to the possibility that something was removed which was needed for antigen activity. It was shown that serological specificity was retained in the extracted membranes, but was only manifest after the addition of concentrated extract, which could come from erythrocytes of any type or from extracts of other mammalian tissues (12). ABO, M, and N activities were not affected by this procedure. These preliminary investigations led to purification studies of the filtrate in which it was found that phosphorylcholine appeared to be the most active lipid, which, when added back to the erythrocyte membranes in a variety of ways, restored the Rh antigen activity.

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to between 80 and 75% of that of the original lyophilized membranes (13).

These present investigations relate to the binding of labeled lipids to the 1-butanol-extracted membranes and subsequent regeneration of Rh antigen activity. In addition, an extensive study was made of the capacity of various classes of lipids to regenerate the Rh antigen activity of the 1-butanol-extracted membranes. In this way, it was hoped to find out what degree of lipid specificity was involved, what kind of binding was significant in the interaction of the lipid and the membranes, and, hopefully, to clarify how the binding of the relevant lipids led to regeneration of serological reactivity. This membrane lipid requirement for active antigenic activity has significant parallels in lipid requirements, of varying degrees of specificity, for both mammalian insoluble membrane enzyme activities (14) and for soluble bacterial cell wall derived enzymes (15). Whether this present membrane lipid requirement is also related to the so-called auxiliary lipids of soluble antigen preparations (16) remains to be determined.

METHODS

Serological

Anti-Rh antisera were obtained from Hyland Division, Travonol Laboratories, Inc. (Yonkers, N. Y.) and from Behring Diagnostics, Inc. (Woodbury, N. Y.), and were checked for specificity but were not further purified prior to use. The measurement of antigen activity was surveyed primarily by the specific adsorption or consumption of antibody by erythrocyte membranes or their insoluble derived products. The membranes (usually 30 to 35 mg) in dry form were weighed out, and standardized amounts of antisera were added for incubation and subsequent centrifugation. The amount of antibody remaining in the supernatant after consumption was compared with controls consisting of unextracted membranes, extracted membranes with no lipid added, membranes with lipid under study, and untreated antisera. Trypsinized human Rh-positive erythrocytes were used (17). Dilution of the antisera was accomplished with a Hamilton 100-μl graduated syringe with measured volumes of antiserum over a 10-fold range. No nonspecific reactions were encountered with this procedure. However, the unusual precision of attempting to quantitate with a hemagglutination end point is implicit (18). The end point of hemagglutination was compared with a "standard" curve prepared by incubating increments of lyophilized unextracted membranes with the standard anti-Rh antibody and measuring residual unabsorbed antibody. The dilution of antisera, with a Hamilton micropipette delivering from 5 to 100 μl of antisera and corresponding amounts of buffer, permitted a modest improvement over the conventional dilution by halves technique, in quantitation of residual antibody and, therefore, antigen activity. However, even with this modification, lipids could differ by 20% or more in their capacity to regenerate the Rh antigen activity without this difference becoming obvious.

A second serological procedure with heat elution was also employed (19). The 1-butanol-extracted membranes with and without the addition of lipid were incubated with suitable concentrations of antibody solution. After washing with phosphate buffered 0.9% NaCl solution, heat elution was carried out by incubation at 58° for 15 min. The supernatant, after centrifugation, was studied for anti-Rh antibody activity against trypsinized Rh-positive erythrocytes.

Erythrocyte Membrane Preparation

Human erythrocytes recently outdated for human use were obtained from the Red Cross, washed with decreasing concentrations of phosphate buffer at pH 7.4 and then lyophilized. Extraction was carried out at 4° with 1-butanol which had been freshly distilled (13). The extractions were repeated three to four times on a given amount of membranes, and between extractions the residue was reduced in a nitrogen stream to close to dryness using negative pressure. After vacuum sublimation to remove residual 1-butanol, the membranes were ground to a very fine powder, with an agate mortar and pestle. The membranes could be maintained by storing over phosphorus pentoxide at 4°, at least up to several weeks after extraction.

Lipid Phosphorus Determination

Lipid phosphorus was determined by the methods of Svanborg and Svennerholm (20). Lipids were obtained from Applied Science Laboratories (State College, Pa.).

Binding of Lipids to 1-Butanol-

extracted Membranes

Binding Conditions

In Benzene Solution—The dissolved lipid, either labeled or unlabeled, was added to the 1-butanol-extracted membranes, allowed to incubate with gentle agitation for 1 hour at room temperature, and the benzene removed either by vacuum sublimation or by blowing ultrapure nitrogen.

By Methanol-Ether Dispersion—This was done by dissolving the lipids in methanol-diethyl ether (1:1, v/v) and then adding buffer or water dropwise so that the total organic solvent concentration was reduced to 5% (21).

By Ultrasonication—This was performed in ice-cooled chambers, with a microtip at an output of 50 watts from the Sonifier Cell Disruptor, model W 185 D (Heat Systems, Ultrasystems, Inc., Plainview, N. Y.). The details of time will be discussed under "Results." The product (22), known as "liposomes," has been the subject of a great deal of investigation as a protein-free continuous model membrane (23). These aggregates are not true micelles, have no measurable critical micellar concentration, and require at least one unaturated ω fatty acid (24).

Measurement of Lipid Bound

The 14C-labeled lipids were obtained from Applied Science Laboratories and New England Nuclear Corp. (Boston, Mass.) and were all tested by thin layer chromatography for both chemical and radiochemical purity. Further isolation and purification was done by isolating the spots and counting them and the remaining portion of the chromatogram with suitable phosphors in a liquid scintillation counter together with appropriate standards and controls. The phosphatidylcholine was found to be less than 80% pure on arrival and was, therefore, purified to 99+ % by elution from Chromar® paper (Mallinkrodt, St. Louis, Mo.) with the fluorescence pattern as marker. The visualized spots were removed by cutting out the paper and extracting with chloroform-methanol. Measurement of lipid binding was carried out both by measuring the supernatant after
suitable incubation of the membranes with the labeled lipid and centrifugation, as well as by benzene washing and subsequent extraction of the membranes with the chloroform and methanol. The fall in radioactivity of the supernatant could only be carried out with the benzene-binding experiments, since centrifugation in the other systems usually resulted in a small amount of sedimentation of lipid aggregates which therefore mixed with the membranes. The influence of concentration of “free” lipid on the amount bound was measured in all systems. The results were plotted in terms of micromoles of lipid bound per mg of membranes versus the unbound concentration, which was arrived at either directly or indirectly. Further calculations from this type of binding experiment were limited by the fact that under none of these experimental binding conditions was the lipid actually in completely unassociated form. It is known that in nonpolar solvents such as benzene there is association of phosphatidylethanolamine molecules (28). It is, in addition, known that the water-swollen phospholipid is in the form of bilayer membrane-like vesicular particles which are birefringent under polarizing microscopy (26). In the case of the methanol-ether-water dispersion it is also likely that some type of membrane-like particles were formed, the difference in this case being that the molecules of organic solvent also participated in the formation of these particles, which are not birefringent. Where lipids would not dissolve in benzene the problem arose that any failure to regenerate Rh antigen activity might be a function of the physical form of the lipid such that a sufficient “free” concentration could not be achieved. Since the more polar organic solvents, such as methanol, ethanol, and 1-propanol, in which phosphatide molecules are known to be unassociated (25), always resulted in irreversible loss in Rh activity, these solvents could not be used. Mild warming to 37° was used for some of the saturated lipids and the more extensive ultrasonication for others. A further maneuver was resorted to in order to test the efficacy of the sparingly benzene-soluble, saturated, synthetic phospholipids. This procedure took advantage of the known effect of lipid-lipid interaction resulting in a very soluble lipid favoring the solubilization of a less soluble lipid (27). Small, suboptimal amounts of unsaturated egg or bovine heart lecithins were incubated with increasing amounts of distilled lecithins and other phospholipids to check for augmented binding and antigen regeneration.

Although direct evidence was lacking, it seems most likely that the site of the binding lipid is membrane protein. Chloroform-methanol extraction of the 1-butanol-extracted membranes showed a small amount of residual phospholipid in these membranes, and it seems very unlikely that this small amount, amounting to slightly over 1% of extracted membrane, could serve as a focus for binding the much larger amounts of phospholipid which were needed for optimal Rh antigen regeneration. The binding of phosphorylcholine was also measured with similar techniques. All lipids were restudied just before use by appropriate thin layer techniques to ensure identification and purity. A technique of silver nitrate-impregnated silica gel (28) was also used for analysis of the distribution of the label in lecithins containing fatty acids of differing degrees of saturation. This analysis showed that only 10% of the label was in disaturated lecithins.

Preliminary kinetic experiments to measure the rate of uptake and release of labeled lipids from the 1-butanol-extracted membranes indicated that the quantitatively most significant time was in the first 3 to 5 min. Since available techniques did not permit such rapid study, these experiments were not pursued.

RESULTS

The complete loss of Rh antigen activity after 1-butanol extraction of the Rh-positive membranes was confirmed, as well as the regeneration of 50 to 75% of the original activity of the unextracted membranes after the addition of egg phosphatidylcholine. This regeneration depended in its extent on the amount of lecithin added back, as well as in the manner in which this was done. Some regeneration was noted with as little as 0.1 mg of lecithin per 35 mg of membranes, but maximum activity was not achieved until at least 3 mg were added back (Fig. 1).

Anti-Rh Antibody Elution Studies—In order to confirm the validity of the more convenient consumption experiments, anti-Rh (D) antibody was incubated with the Rh-positive 1-butanol-extracted membranes, and after washing with phosphate-buffered 0.9% NaCl solution and heat elution at 56°, the product was centrifuged and the supernatant studied for anti Rh activity. Active antibody was found in significant titer but not on elution of the membranes to which no lecithin had been added back. This confirmed that the binding of antibody to the Rh-positive 1-butanol-extracted membranes required the lipid present on the membranes.

Excluding Nonspecific Hemagglutination Inhibition—Because high concentrations of lipid-containing substances have been noted in the past to have nonspecific inhibition effect (29), the lipids used in these experiments were all studied without any membranes present to check for inhibition of hemagglutination. The lipids were studied in concentrations up to twice that used for the membrane-binding experiments, and neither lecithins, phosphatidylethanolamine, nor various fatty acids had any effect of inhibiting hemagglutination in the present system.

Binding Conditions—The binding of the lecithin in benzene solution with subsequent removal of the reagent was the most convenient method. But, regeneration was slightly less than with the two methods in which the lecithin was presented to the membranes in the form of a sonicated aqueous dispersion of
with 5.0 ml of chloroform-methanol. The bound lipid was then washed with 20 ml of benzene and then extracted twice. The samples were lyophilized in 1.0 ml of sonicated aqueous solution. For experiments in which the lipids were incubated in benzene solution, similar amounts of membranes were used, and the phospholipid or fatty acid was dissolved in 1.0 ml of chloroform-methanol, 1:4. The bound lipid was calculated from the radioactivity of this extract.

![Figure 2](http://www.jbc.org/) Binding of labeled lipids to 1-butanol-extracted membranes. Δ, [U-14Cllecithin in sonicated aqueous dispersion; △, [U-14Cllecithin dissolved in benzene; ○, [14C]oleic acid dissolved in benzene. Varying amounts of 1-butanol-extracted membranes, from 5 to 100 mg, were used. Phospholipid, 1 µmole, was added in 1.0 ml of sonicated aqueous solution. For experiments in which the lipids were incubated in benzene solution, similar amounts of membranes were used, and the phospholipid or fatty acid was dissolved in 1.0 ml of benzene. The samples were lyophilized after incubation for ½ hour at room temperature. The specimens were then washed with 20 ml of benzene and then extracted twice with 5.0 ml of chloroform-methanol, 1:4. The bound lipid was calculated from the radioactivity of this extract.

![Figure 3](http://www.jbc.org/) Duration of sonication and amount of lecithin bound. Aqueous dispersion of [U-14Cllecithin was prepared, and 0.25-µmole aliquots in 1 ml of water were removed after various periods of sonication, incubated with 35 mg of 1-butanol-extracted membranes for 1 hour at 24°, and then lyophilized. After washing with benzene, the samples were extracted with 5.0 ml of chloroform-methanol, 1:4, and calculation of the amount bound was made from the radioactivity of the extract.

Fig. 2. Binding of labeled lipids to 1-butanol-extracted membranes. Δ, [U-14Cllecithin in sonicated aqueous dispersion; △, [U-14Cllecithin dissolved in benzene; ○, [14C]oleic acid dissolved in benzene. Varying amounts of 1-butanol-extracted membranes, from 5 to 100 mg, were used. Phospholipid, 1 µmole, was added in 1.0 ml of sonicated aqueous solution. For experiments in which the lipids were incubated in benzene solution, similar amounts of membranes were used, and the phospholipid or fatty acid was dissolved in 1.0 ml of benzene. The samples were lyophilized after incubation for ½ hour at room temperature. The specimens were then washed with 20 ml of benzene and then extracted twice with 5.0 ml of chloroform-methanol, 1:4. The bound lipid was calculated from the radioactivity of this extract.

larger particles. Problems were found due to binding of the lecithin to glass surfaces; such binding was prevented by silanization (30). The binding of the lecithin in benzene solution indicates that the equilibrium is strongly in favor of the bound phase. This was confirmed by washing the membrane-lipid complex with benzene, drying, and testing for maintenance of regenerated antigen activity. This activity was indeed found to be preserved after the benzene wash. This was also true if the lecithin was bound using a method of dispersion in an aqueous phase. Thus, benzene washes could be used to remove the unbound lipid in the liposome-binding experiments with minimal disturbance of the binding equilibrium.

The labeled lipid-binding experiments show that the difference in binding in aqueous dispersion compared with that in benzene solution is a function of the unbound concentration, and at the lower regions no difference can be seen (Fig. 2). The binding of oleic acid was also compared with that of lecithin, and the extent of uptake was less under conditions of the same free concentration. However, with higher concentrations more bound, reaching the level of bound lecithin which was required for optimal Rh antigen regeneration. The amount of antigen regeneration with the aqueous lecithin preparation was somewhat greater than with the lipid dissolved in benzene. However, this difference could not be well quantified due to the imprecision of hemagglutination end point. There was no regeneration using oleic acid, in spite of its binding to the 1-butanol-extracted membranes.

The effect of sonication was measured as a function of time at constant power output (50 watts). From Fig. 3 it is clear that the maximum lecithin uptake only occurs after 2 min of sonication. Since the total concentration of lipid did not change as a function of time, it is apparent that particle size has had a significant effect on the uptake of lipid onto the membranes. It is known that increasing sonication time results in a smaller particle size which approaches a bilayer vesicular structure (22). With sonication for 2 min or more it was found that the extent of binding of lecithin was about the same as that of lecithin taken up in the methanol-ether dispersion, but regeneration of Rh antigen activity was better with the sonicated phospholipid.

**Effect of Phosphorylcholine on Lecithin Uptake**—These experiments were designed to show whether lecithin uptake could be decreased by the presence of high concentrations of phosphorylcholine, with the idea that if electrostatic interaction was significant in the binding of lecithin to the membranes, this might be reduced by presenting an excess of the identical polar portion of the lecithin molecule at the same time. Up to a 10-fold concentration ratio of phosphorylcholine to lecithin was used, and only a very slight fall in the amount of lecithin bound was noted. In addition to this, the binding of phosphorylcholine was measured without lecithin by incubating in aqueous solution at pH 7.4, centrifuging the 1-butanol-extracted membranes, and measuring the fall in radioactivity of the supernatant. Using 1 µmole of phosphorylcholine (corresponding to the concentration of lecithin normally used) only a very slight fall in supernatant was seen. These experiments would suggest that the majority of the binding of the lecithin to the membranes is probably not electrostatic in type. A further possibility to be considered was that the presence of the polar group may affect the binding of the fatty acid. This was tested in the following way. [14C]Oleic acid and phosphorylcholine were used in concentrations such that, if they were present as residues in lecithin molecules, both binding and regeneration would be optimal. It was found first that [14C]Oleic acid binding was not increased in the presence of phosphorylcholine, and second, that Rh regeneration did not take place when both were added at the same time.

**Lipid Classes and Rh Antigen Regeneration**—An extensive series of lipids was studied in molar concentrations up to 2- to 3-fold the optimum concentration of lecithin. And, since some effect of egg lecithin was measurable at one-tenth the optimum concentration,
the sensitivity of the screening tests was at a level of about one-sixtieth of the activity of lecithin. Cholesterol and its esters had no effect (Table I). The even-numbered saturated fatty acids from C16 to C20 were all studied and found to have no effect. In addition, the odd chain fatty acids were studied and again no effect was seen. The methyl and ethyl esters of many of these were tried, and again no effect was found. A number of unsaturated fatty acids were studied and only oleic acid was found to have a trace of effect in some experiments with very large amounts. Triolein had no effect. Phosphatidylethanolamine

Table I
Nonphosphorous-containing lipids tested for Rh regeneration

These lipids were studied for their capacity to regenerate the Rh antigen activity of Rh-positive 1-butanol-extracted membranes with twice the molar concentration necessary for optimal regeneration with egg lecithin. The lipids were dissolved in 1 ml of benzene, added to 35 mg of membranes, incubated for 1 hour at 24°, and the benzene removed. Parallel specimens with unextracted membranes, and extracted membranes alone and with egg lecithin, were included in each experiment. Only a trace of activity was found with oleic acid.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Rh regeneration %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straight chain, even number, fatty acids, C24 to C28</td>
<td>None</td>
</tr>
<tr>
<td>Odd number, fatty acids, C12 to C20</td>
<td>None</td>
</tr>
<tr>
<td>Methyl esters, straight chain, even number, fatty acids, C12 to C20</td>
<td>None</td>
</tr>
<tr>
<td>Ethyl esters, straight chain, even number, fatty acids, C12 to C20</td>
<td>None</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>None</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>None</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>None</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>None</td>
</tr>
<tr>
<td>Triolein</td>
<td>None</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>None</td>
</tr>
<tr>
<td>Cholesterol oleate</td>
<td>None</td>
</tr>
<tr>
<td>Cerebroside</td>
<td>None</td>
</tr>
</tbody>
</table>

Table II
Phospholipids and Rh antigen regeneration

These phospholipids were studied for antigen-regenerative capacity both in benzene solution and in the form of sonicated aqueous dispersions (liposomes) at the same concentration as that resulting in maximal regeneration with egg or bovine heart lecithin (0.12 μmole per mg of membranes).

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Rh antigen regeneration: percentage of that of egg lecithin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylethanolamine (mixed fatty acids)</td>
<td>90-100</td>
</tr>
<tr>
<td>Diposphatidylglycerol (mixed fatty acids)</td>
<td>60</td>
</tr>
<tr>
<td>Phosphatidylserine (mixed fatty acids)</td>
<td>25</td>
</tr>
<tr>
<td>Sphingomyelin (mixed, but not unsaturated fatty acids)</td>
<td>0</td>
</tr>
<tr>
<td>Phosphatidylcholine (dipalmitoyl)</td>
<td>0</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (dipalmitoyl)</td>
<td>0</td>
</tr>
</tbody>
</table>

was studied and found to be approximately as effective as lecithin. Phosphatidylethanolamine was clearly less effective than lecithin, but did have some effect, as did diposphatidylglycerol (Table II).

Fully Saturated, Synthetic Lecithins—Dipalmitoyl phosphatidylcholine, both DL and L forms, was found to have no effect in Rh regeneration. Because of low solubility in nonpolar organic reagents, the question arose as to whether the lack of regenerative capacity was due merely to the unfavorable form in which it could be presented. These lecithins were made optically isotropic in benzene solution after a small amount of ultrasound, but even with this and with heating, no regeneration of activity was found. This was also true when the saturated lecithins were added back in methanol-ether aqueous dispersion. A further method was used to attempt to answer this question more clearly, taking advantage of known increased solubilization due to lipid-lipid interaction. Suboptimal amounts of unsaturated lecithins, 0.25 mg/30 mg of membranes, were added in benzene solution together with increasing amounts of dipalmitoyl lecithin. This succeeded in solubilizing most of the saturated lecithin. No augmentation of antigen activity was found. This suggested further that the failure to regenerate the antigen activity was not due to an unfavorable solubility situation. This was confirmed by measuring the binding of the saturated lecithin to the 1-butanol-extracted membranes (Fig. 4). That

Fig. 4. Binding (▲) to, and Rh antigen regeneration (○) of 1-butanol-extracted membranes with dipalmitoyl lecithin. Each sample contained 20 mg of 1-butanol-extracted membranes, to which were added 0.3 μmole of egg lecithin and varying amounts of dipalmitoyl lecithin from 0 to 3.6 μmole in a total volume of 0.3 ml of benzene. This was followed by a 15-min incubation at 21°, after which the tubes were dried with nitrogen. For the Rh antigen regeneration studies, anti-Rh antisera were added, and after incubation and centrifugation, studied for augmented Rh antigen regeneration above the slight level found due to the small amount of egg lecithin. For the binding studies, the samples were washed twice with 10 ml of benzene and extracted twice with 5.0 ml of chloroform-methanol, 1:4. The lipid phosphorus of the extracts was measured, and the amount in each extract minus the amount in the first sample having no added dipalmitoyl lecithin (to correct for egg lecithin bound and residual extractable membrane phosphorus) gave the amount of dipalmitoyl lecithin bound.
binding did indeed take place was established, and this could be pushed to reasonably high levels, but no increased regeneration of the Rh antigen activity resulted. Similarly, saturated phosphatidylethanolamines had no regenerating effect.

**DISCUSSION**

A graded regeneration of the Rh antigen activity of human Rh-positive lympholized erythrocyte membranes abolished by 1-butanol extraction has been shown. Specificity is apparent at several levels. Only phospholipids were active. Phosphatidylserine and cardiolipin were inferior to lecithin and phosphatidylethanolamine. Lecithins with fully saturated fatty acids did not function. It is also apparent that many lipids bind to the 1-butanol-extracted membranes but do not result in regeneration. Thus, binding is not enough. A further factor must be required, and this appears to be related to the nature of the fatty acid requirement. However, the fatty acids alone did not regenerate the activity, and phospholipids of precisely defined unsaturated fatty acid composition are difficult to prepare.

There is much current interest in the structure of membranes and also in various enzymes which are intimately linked, both anatomically and functionally to membranes (31). The relation between membrane lipids and membrane proteins is one of the basic problems that has not been completely resolved. One of the serious difficulties is that isolation or even study, for example, with electron microscopy may involve changes from the normal in vivo situation (32). It has been pointed out that when the lipid and protein constituents from lipoproteins (33), and probably also from membranes, are separated and then allowed to recombine, there is no assurance that they are binding in the same way in vivo, although this may be thermodynamically most likely in isolated systems. The present system allows study of the binding of lipids from free solution or in the form of liposomes to the 1-butanol-extracted membranes, and at the same time the regeneration of Rh antigen activity can be followed. Because of this correlation one can be reasonably sure that the binding of the phospholipid takes place in a "physiological" way, as opposed to the situations where binding of lipids and protein are not also related to some measurement of biological activity.

In the case of the Rh system, the lyophilization step has essentially destroyed the red cell membranes in their functioning capacity as membranes. On the other hand, as far as can be ascertained, the Rh antigen activity is not diminished through this procedure. The extraction of the lyophilized erythrocyte membranes and the 1-butanol removed essentially no protein, in contradistinction to the more frequently employed aqueous 1-butanol procedure suggested by Morton (34) and more recently described by Rega et al. (3) and Poulik and Bron (35), who found that the aqueous 1-butanol procedure applied to human erythrocyte cytes results in products which do not manifest any Rh antigen activity.

The relative contribution of hydrophobic versus electrostatic forces in the binding of lipids to each other and to other types of molecules is often difficult to assess in the biological systems of any degree of complexity (36). Klots has even questioned whether such a breakdown is meaningful (37). The binding of lecithin has been clearly shown to take place via electrostatic interactions to glass surfaces (30) and to salt crystals (38). On the other hand, it is likely that lecithin can bind hydrophilically to protein in membranes (39).

These present studies do not explicitly define what it is in the 1-butanol-extracted erythrocyte membranes which binds the phospholipids. Indirect evidence previously alluded to would suggest that what determines the serological specificity of the Rh antigens is a membrane protein requiring free sulfhydryl groups for activity. This protein has never been recognized in soluble form. It is possible that previous attempts to solubilize the red cell Rh antigens have not been as successful, not because of the serologically specific protein had not been solubilized, but because the role of lipids was not known. These attempts at solubilization of the protein portion of the antigen are complicated by the known sensitivity of the Rh antigens to conditions often used in isolation procedures. The possibility cannot be entirely excluded that the Rh antigens in toto will never be found in free solution, because a sufficiently large membrane structure that can provide the configuration necessary for antigen activity may never allow for true solubilization. Even the types of "supermolecule" derived by disaggregation of erythrocyte membranes by sodium lauryl sulfate treatment did not have any residual Rh antigen activity (11). However, this pessimistic prediction is more or less a diagnosis of exclusion and, hopefully, current attempts to solubilize membrane protein having antigen activity in conjunction with lipids will be successful.

**REFERENCES**
