Correction. In the article "Fetal mouse hearts: A model for studying ischemia" by J. S. Ingwall, M. DeLuca, H. D. Sybers, and K. Wildenthal, which appeared in the July 1975 issue of Proc. Nat. Acad. Sci. USA 72, 2809-2813, the authors have requested the following changes. On page 2810 in the first line of the legend for Table 1, the control ATP levels should be 41 ± 4.0 nmol/mg of protein instead of μmol/mg. The same change should be made in the last line of the caption for Fig. 1 on page 2811.

Correction. In the article "Apparent dependence of interactions between cytochrome b5 and cytochrome b5 reductase upon translational diffusion in dimyristoyl lecithin liposomes" by P. Strittmatter and M. J. Rogers, which appeared in the July 1975 issue of Proc. Nat. Acad. Sci. USA 72, 2658-2661, the authors have requested the following changes. The amounts of lecithin are incorrectly indicated in Table 1 and Fig. 1 on page 2659. In all cases the amount of lecithin should read μmol, not mmol.

Authors' Statement on the Isolation of mRNA Coding for Immunoglobulin Heavy Chain

The method (1) which we previously described for the specific purification of mRNA coding for immunoglobulin heavy chain (mRNA-H) has proved to be irreproducible. A specific interaction between immunoglobulin and mRNA-H was postulated as an explanation of the translational control of H chain synthesis (2, 3). We reported that a considerable purification of mRNA could be obtained by interacting RNA from immunoglobulin producing cells with immunoglobulin and precipitating with anti-immunoglobulin. During the past year in both Los Angeles and Glasgow we have been unable to reproduce the purification of mRNA-H. Translation of precipitated RNA does give rise to H chains, identified immunologically, but we have not obtained supporting evidence for specific enrichment of mRNA-H in the precipitated fraction.

A test of the hypothetical interaction between mRNA-H and its translation product must now await isolation of mRNA-H by other routes. The interpretation of experiments in which mRNA isolated by the precipitation method (4-8) was used must be reconsidered wherever they depend on the purity of the putative mRNA-H.

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Apparent dependence of interactions between cytochrome \( b_5 \) and cytochrome \( b_5 \) reductase upon translational diffusion in dimyristoyl lecithin liposomes

(membrane-bound enzymes/lipid–protein interactions)

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ABSTRACT  Dimyristoyl lecithin liposomes, containing cytochrome \( b_5 \) reductase (NADH:ferricytochrome \( b_5 \) oxidoreductase, EC 1.6.2.2) and varying amounts of cytochrome \( b_5 \), were used to measure flavoprotein catalysis alone and catalysis requiring electron transfer between the reductase and cytochrome as a function of temperature. Whereas flavoprotein catalysis showed a simple linear temperature dependence in an Arrhenius plot, the reaction involving electron transfer between the two bound enzymes showed a marked, 4-fold, change in rate at the crystalline–liquid crystalline phase transition of the hydrocarbon chains of the lecinthin vesicles and a second, minor change involving the minor transition. These data represent strong evidence that protein–protein interactions in this membrane model system are dependent upon translational diffusion of nonpolar segments of the proteins in the hydrocarbon region of the phospholipid bilayer.

The initial characterization of the binding of cytochrome \( b_5 \) to microsomes (1) led us to propose a model for cytochrome interaction with cytochrome \( b_5 \) reductase (NADH:ferricytochrome \( b_5 \) oxidoreductase, EC 1.6.2.2) in which both proteins are randomly distributed on the outer side of the vesicles and undergo translational diffusion in the plane of the membrane prior to catalytic interaction. The amphipathic structure of the heme protein and flavoprotein results, in each case, in attachment by a nonpolar peptide segment, thus orienting the hydrophilic, catalytic portion of each molecule, containing the functional coenzyme, to the membrane–solution interface (2, 3). Subsequent examination of both cytochrome and reductase binding to lipid vesicles, as well as studies on the kinetics of the interactions of the two proteins, in either microsomes on egg lecithin vesicles, have provided further data consistent with this hypothesis (4–7).†

We describe here a test for protein–protein interactions which are dependent upon lateral diffusion of the nonpolar peptide portions of these molecules in the lipid bilayer. It is based upon the binding of the two enzymes to dimyristoyl lecithin liposomes. Since these vesicles show a sharp phase transition at 23° (8, 9), it was possible to compare the temperature dependence of the catalytic activity involving only the flavoprotein, i.e., NADH:ferricyanide reductase (6), to that of the overall cytochrome \( c \) reductase activity involving a sequence of electron transfer from NADH to reductase to cytochrome \( b_5 \) and, finally, to a large excess of the soluble electron acceptor, cytochrome \( c \) (6). Only in the latter case, which requires reductase–cytochrome \( b_5 \) interaction on the liposome, and at relatively low concentrations of both bound proteins, did an abrupt increase in activity accompany the crystalline–liquid crystalline phase transition of the lipid. A fluid bilayer, permitting protein diffusion, therefore appears to be required for catalytically productive interactions between the reductase and the heme protein.

METHODS AND MATERIALS

Cytochrome \( b_5 \) and NADH:cytochrome \( b_5 \) reductase were prepared from steer liver as described previously (2, 3) and stored at –20° in Tris–salt buffer (0.01 M Tris-acetate, pH 8.1, containing 0.1 M NaCl, 0.2 mM EDTA, and 0.02% sodium azide). Disaggregation of frozen and thawed reductase was achieved by incubating the enzyme at 25° for 5 hr in Tris-salt buffer as described previously†. NADH and horse heart cytochrome \( c \) were products of Sigma Chemical Co.

The methods for determining the concentration of cytochrome \( b_5 \) and the reductase, NADH:ferricyanide reductase activity, and NADH:cytochrome \( c \) reductase activity have been described (6). Phospholipid was determined by the method of Chen et al. (10).

Liposomes were prepared from synthetic \( \beta \gamma \)-dimyristoyl \( L \alpha \)-lecithin (Calbiochem) by a modification of the method of Huang (11) by sonicking 25–40 mg of the lecithin, suspended in 2.5 ml of Tris-salt buffer in a 15 ml Corex tube, at 26° for 40 min under a stream of nitrogen. This temperature, 26° rather than 2° in Huang’s original procedure, was chosen to exceed the transition temperature of this lipid. The probe tip was immersed to a depth of 5 mm. This sonication procedure resulted in the conversion of 85–90% of the lecithin to liposomes (11). Centrifugation of the vesicles at 100,000 X g for 30 min removed most of the lamellar material remaining after sonication. Under these conditions, approximately 5% or less of the lecithin was converted to lysolecithin, free fatty acids, and other products as noted by Hauser (12) for other types of lecithins. The amount of breakdown was estimated by the density of rhodamine B staining of silica thin-layer plates after chromatography using chloroform:methanol:7 N NH₄OH (230:90:15). The concentration of liposomes is reported as the molar concentration of liposomal phospholipid. Liposomes were stored under nitrogen at 26° and used for no longer than 4 days.

All incubations, column chromatography on Bio-Gel A-1.5m (Bio-Rad), and catalytic assays were performed in the same Tris-salt buffer to avoid changes in ionic strength and bacterial contamination by inclusion of sodium azide.

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Abbreviation: Tris-salt buffer, 0.01 M Tris-acetate, pH 8.1, containing 0.1 M NaCl, 0.2 mM EDTA, and 0.02% sodium azide.

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† M. J. Rogers and P. Strittmatter, J. Biol. Chem. in press.
Table 1. Binding of cytochrome b₅ and reductase to dimyristoyl lecithin liposomes

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Isolated liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyt b₅/lecithin, nmol/mmol</td>
</tr>
<tr>
<td>Exp.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.86</td>
</tr>
<tr>
<td>2</td>
<td>2.6</td>
</tr>
<tr>
<td>3</td>
<td>22.8</td>
</tr>
</tbody>
</table>

Cytochrome b₅ (11–78 μM) was incubated with 4.5–11.8 mM phospholipid as liposomes for 10 hr at 20°C in Tris-salt buffer. After addition of reductase to a final concentration of 4–5.5 μM, the incubation was continued for another 1.5 hr. Liposomes containing both cytochrome b₅ and reductase were then isolated by gel filtration as illustrated in Fig. 1. Analysis of the peak liposome fraction in each experiment is shown since this material was used in the following experiments.

RESULTS

Table 1 shows the incubation conditions used to obtain liposome preparations containing a greater than 20-fold variation in cytochrome b₅ concentration with a relatively constant amount of the reductase. In Exps. 1 and 2 virtually all of the reductase added was bound, but at the higher cytochrome concentrations (Exp. 3), extensive initial binding of the heme protein limited the subsequent overall flavoprotein binding to 60–70%. Fig. 1 illustrates the effectiveness of the gel filtration procedure for the separation of liposomes containing bound proteins from free protein by the method employed with egg lecithin vesicles. The peak fraction containing the highest phospholipid concentration was used, in each case, for the kinetic experiments below.

Electron micrographs of liposomes, with or without bound protein and negatively stained with phosphotungstate, showed relatively uniform vesicles approximately 250–400 Å in diameter. As Fig. 2 shows, even at the highest concentration of protein, the liposomes appear to be relatively undistorted and uniform in size, indicating that the dimyristoyl lecithin vesicles are structurally intact at least over the protein concentration range employed in the incubations shown in Table 1.

Fig. 3 shows the temperature dependence of the ferricyanide and cytochrome c reductase activities of the three liposome preparations. The ferricyanide reductase activities

Fig. 1. Gel filtration of liposomes containing reductase and cytochrome b₅. The incubation mixture from Exp. 2 of Table 1 was applied to a 0.5 × 65 cm Bio-Gel A-1.5m column equilibrated with Tris-salt buffer and 0.4 ml fractions were collected. The fraction containing the highest concentration of lipid and proteins, in this and the other two experiments of Table 1, was used for the kinetic assays and for the electron microscopy.

FIG. 2. Electron micrograph of liposomes containing bound reductase and cytochrome b₅. An aliquot of the liposomes from Exp. 3 of Table 1 was mixed with an equal volume of neutral 2% phosphotungstic acid and applied as a drop to a Formvar-coated grid. Excess fluid was removed with filter paper after 1 min. A Philips EM 300 electron microscope was used at ×100,000. (Curve 1) are the same for all of the preparations and fall on the same straight line on this Arrhenius plot. This would be expected for catalysis involving only the flavoprotein in the reduction of ferricyanide by NADH. These activities represent the maximum velocities of the bound reductase (Vmax) and the slope of the line yields an apparent activation energy of 9000 cal/mol (40 kJ/mol).

In marked contrast, the data for cytochrome c reductase

FIG. 3. Arrhenius plots of NADH:ferricyanide and NADH:cytochrome c reductase activities of liposomes. Aliquots of liposomes described in Table 1 were assayed for NADH:ferricyanide and NADH:cytochrome c reductase activities as indicated in Methods and Materials at the indicated temperatures. The amount of liposome used was chosen to obtain an equal amount of reductase for all assays. Curve 1, NADH:ferricyanide reductase activities of the three liposomal preparations in Table 1, Curves 2, 3, and 4, NADH:cytochrome c reductase activities of liposomes from Exps. 3, 2, and 1 of Table 1, respectively.
activities, which require electron transfer between the bound reductase and cytochrome \( b_5 \), show nonlinear behavior that is dependent upon the cytochrome \( b_5 \) concentration in the liposomes (Fig. 3, Curves 2–4). Since the ferricyanide reductase activity measured \( V_{\text{max}} \) at various temperatures, the observed cytochrome \( c \) reductase activities could be related to the maximum catalytic rate of the reductase (Fig. 4), and compared to the phase transitions for dimyristoyl lecithin vesicles reported by Hinz and Sturtevant (8) and by Tsong (9). The reported main transition involving the hydrocarbon chains is at 23.7–23.8°C, and the minor transition which they attributed to the polar head groups of the lecithin occurs at 12.5–13.5°C. Clearly, even above the phase transitions the catalytic velocities were still dependent upon the cytochrome \( b_5 \) particularly at the lower concentrations (Curves 2 and 3). Furthermore, in all cases the liquid to crystalline transition of the hydrocarbon chains resulted in a marked decrease in the rate of electron transfer at the lowest level of bound heme protein. In addition, transitions involving the glycerophosphate environment are discernible in Curves 2 and 3.

**DISCUSSION**

This system of two oxidative enzymes and a single phospholipid species represents one of the simplest models for the examination of protein–protein interactions in biological membranes. Above the transition temperature of the hydrocarbon chains, the dimyristoyl lecithin liposomes show the same characteristics for binding variable amounts of both enzymes and the kinetic properties that have been observed with microsomal and egg lecithin vesicles (1, 4–7). The retention of these basic properties in the transition from the complex structure of microsomes, to a simple vesicle containing only two proteins and a complex, highly unsaturated lecithin, and, now, to liposomes containing only 14-carbon, saturated fatty acids, suggests that the properties of the model system do reflect the same basic interactions that obtain in situ in endoplasmic reticulum.

It is the combination of the sharply defined crystalline–liquid crystalline phase transitions of dimyristoyl lecithin and the simple assays that distinguishes catalysis involving the flavoprotein alone from catalysis involving the interaction of the reductase with cytochrome \( b_5 \) that permitted a definitive test of the model for random distribution and translational movement (1). The protein and lipid concentration in liposomes, the surface area occupied by lipid head groups and the catalytic segments of the proteins on the outer surface of liposomes, and the assumption that the proteins are randomly distributed indicate that the average distance between a cytochrome \( b_5 \) and a reductase molecule was about 100, 50, and 5 Å in Exps. 1–3 of Table 1, respectively. Even above the transition temperature of the lecithin, a diffusion dependent process is suggested by the approach to \( V_{\text{max}} \) of the cytochrome \( b_5 \) and reductase-dependent activity as the apparent distance between the proteins was decreased to a fraction of the molecular diameters of the proteins.

The most conclusive evidence that translational diffusion of the proteins occurs, however, is the marked decrease in the catalysis involving interactions of the two bound enzymes that occurred upon transition of the hydrocarbon chains to the gel phase below 21–23°C. The effect is minimal when the two enzymes are closely packed, i.e., within approximately 5 Å, but, at greater distances (100 Å) the catalytic interactions were decreased more than 4-fold, to approximately 16% of the \( V_{\text{max}} \) of the flavoprotein. At lower temperatures the minor transition also produced a detectable, but smaller, decrease in the rate of catalysis, until, at 3–6°C, electron transfer between reductase and cytochrome \( b_5 \) was only 11% of the \( V_{\text{max}} \) at the lowest cytochrome concentration. Such a marked dependence of the rate of these protein–protein interactions upon both the fluidity of the phospholipid bilayer and the apparent distance between the molecules is the expected characteristic of a process limited by the rate of diffusion of segments of the proteins that reside in the nonpolar region of the phospholipid bilayer.

The decreases in cytochrome \( c \) reductase activity, reflecting lower rates of reductase to cytochrome \( b_5 \) electron transfer, do not precisely coincide with the transitions reported for pure dimyristoyl lecithin vesicles (8, 9). The hydrocarbon chain transition occurs at 23.7–23.8°C over a 0.2–0.5°C temperature range, and the broader, lower transition over a 2°C range at either 12.5 or 13.5°C. Since our system is a heterogeneous one, containing both proteins and lipids, the slightly broader and lower temperature ranges for the abrupt decreases in the rates of reductase to cytochrome \( b_5 \) electron transfer, i.e., at 19–23°C and at 10–12°C, are not unexpected. It is significant, in this regard, that the bound proteins appear to extend from the hydrocarbon region through the polar head groups to orient the catalytic segments in the aqueous phase (2, 3).

The movement of these proteins, in which various segments experience markedly different environments, would be limited most effectively by the mobilities of the segments which reside in the most viscous portion of the liposomes, the hydrocarbon chains. This is clearly seen in the present data in the dramatic effect of the hydrocarbon chain transition on the rate electron transfer between bound enzymes. With 14-carbon fatty acids, the hydrocarbon region of the bilayer would be only 35 Å thick rather than 45 Å in membranes, such as endoplasmic reticulum, in which 18-carbon
fatty acids predominate. This suggests that the entire span of the nonpolar region of microsomes is not essential for binding of either protein. In dimyristoyl lecithin vesicles the rates of electron transfer at protein concentrations which require separation by about 50 and 100 Å, assuming a random distribution, are less than \( V_{\text{max}} \) of the reductase at 25°, i.e., they are becoming diffusion limited. In Exps. 1 and 2 of Table 1, at calculated distances of 100 and 50 Å, the rates of electron transfer are 0.52 and 0.64/msec, respectively. In microsomes at endogenous levels of cytochrome \( b_5 \) and reductase, the calculated separation is approximately 65 Å and the catalytic rate is 0.46/msec. Translational diffusion constants for these proteins in liposomes are not yet available. However, Cone (13) has reported a value of 3.5 \( \times 10^{-9} \) cm\(^2\) sec\(^{-1}\) for the diffusion constant of rhodopsin, in frog outer rod segments. This value leads to a collision rate for proteins separated by 100 Å of 16/msec. Thus, the translational movement of proteins in membranes appears to be sufficiently rapid to permit the multiplicity of collisions that may be required for a productive event, electron transfer between reductase and cytochrome \( b_5 \).

The amphipathic structure of both proteins also calls attention to the increasing rotational freedom of the polypeptides in progressing from the hydrocarbon, through the head group region, into the aqueous phase. This progressive decrease in viscosity approaching the catalytic portions of both molecules, and the apparent flexibility of the short peptide segment which joins the catalytic and nonpolar peptide regions (2, 3), may play a significant role in facilitating productive interactions between them. With this defined three component, protein–lipid system, nuclear magnetic resonance, electron paramagnetic resonance, and fluorescence probes should now yield definitive data concerning both the environment and the flexibility of each peptide segment of these proteins.

This investigation was supported by Research Grant CM-15924 from the U.S. Public Health Service.