BINDING OF CYTOCHROME b_5 TO BIOLOGICAL AND ARTIFICIAL MEMBRANES

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I. BINDING OF CYTOCHROME $\underline{b}_5$ TO LIVER MICROSOMES

SUMMARY

Cytochrome $\underline{b}_5$, purified from liver microsomes after solubilization with detergents ("d-$\underline{b}_5$"), could bind effectively *in vitro* to liver microsomal membranes. The binding was more efficient at 37°C than at 0°C, and proceeded at a relatively slow rate. The cytochrome once incorporated could not be removed by repeated washing with 0.3 M KCl or 10 mM EDTA. The bound cytochrome was reducible by NADH *via* NADH-cytochrome $\underline{b}_5$ reductase. Trypsin-solubilized cytochrome $\underline{b}_5$ ("t-$\underline{b}_5$"), which has been shown to lack a hydrophobic region present in the d-$\underline{b}_5$ molecule, could not be inserted into the microsomal membranes. It is concluded that externally added d-$\underline{b}_5$ can be firmly attached to micosomes through a hydrophobic interaction between the hydrophobic region of the cytochrome molecule and the membrane matrix.

INTRODUCTION

Cytochrome $\underline{b}_5$ can be readily solubilized from liver microsomes with trypsin and other proteases and highly purified (1). However, it is likely that the cytochrome preparations thus obtained are nothing but proteolytically modified products of the native protein. To check this possibility, Ito and Sato (2) solubilized the hemoprotein with detergents so as to avoid cleavage of peptide bonds and purified it to near homogeneity. The cytochrome $\underline{b}_5$ preparation thus obtained ("detergent-solubilized cytochrome $\underline{b}_5$" or "d-$\underline{b}_5$") was found to be an amphipatic protein consisting of a hydrophilic region containing protoheme and a hydrophobic portion and, because of the hydrophobic moiety, exist as an oligomer in aqueous solution. On the other hand, cytochrome $\underline{b}_5$ purified after solubilization by tryptic digestion ("trypsin-solubilized cytochrome $\underline{b}_5$" or "t-$\underline{b}_5$") was shown to be a fragment produced from the native protein (d-$\underline{b}_5$) by proteolytic removal of the hydrophobic

moiety. Spatz and Strittmatter (3) later purified d-b_5 by a different metod and reached essentially the same conclusion; they also reported that d-b_5 has an additional peptide segment consisting of 40-44 mainly hydrophobic amino acid residues, which are lacking in t-b_5.

The possible functional significance of the hydrophobic moiety in the d-b_5 molecule has been investigated. Thus, Okuda *et al.* (4) showed that d-b_5 interacts with a preparation of native NADH-cytochrome b_5 reductase much more effeciently than t-b_5 does. Moreover, Shimakata *et al.* (6) demonstrated that only d-b_5, but not t-b_5, is capable of reconstituting of an active stearoyl-CoA desaturase system with preparations of NADH-cytochrome b_5 reductase and cyanide-sensitive factor.

Apart from such functional importance, the hydrophobic region in the d-b_5 molecule has been suggested to have a structural implication (2,3,5). It seems highly likely that the hydrophobic moiety is involved in the tight binding of the cytochrome to the microsomes. According to the fluid mosaic model of cellular membranes described by Singer and Nicolson (7), an amphipathic protein such as d-b_5 is a typical membrane protein which is intercalated into the membrane matrix by a hydrophobic interaction. If this is really the case, it is expected that exposure of d-b_5 to microsomes will result in the insertion of the cytochrome molecules into the membrane structure. The present study was undertaken to examine this expectation experimentally.

This report describes that binding *in vitro* of d-b_5, but not t-b_5, to liver microsomes does occur, as expected, and that the binding of d-b_5 to the microsomal membranes involves a hydrophobic interaction between the hydrophobic region of the protein and the membrane matrix.

EXPERIMENTAL PROCEDURE

Preparation of liver microsomes. Rabbit liver microsomes were prepared with 0.15 M KCl as homogenizing medium and washed with 0.15 M KCl containing

10 mM EDTA (pH 7.0) and then with 0.1 M phosphate buffer (pH 7.5) as described by Omura and Takesue (8). The micosomal preparations thus prepared contained about 1 nmole of cytochrome $\underline{b}_5$ per mg of protein.

Purification of Cytochrome $\underline{b}_5$. The d-$\underline{b}_5$ preparations used (43-47 nmoles per mg of protein) were purified from rabbit liver microsomes by the method of Spatz and Strittmatter (3); they were practically free of phospholipids and dtergents (3). The t-$\underline{b}_5$ preparations (70-79 nmoles per mg of protein) were also purified from rabbit liver microsomes as described by Omura and Takesue (8). Both d-$\underline{b}_5$ and t-$\underline{b}_5$ preparations were essentially homogeous upon 15% cross-linked polyacrylamide gel electrophoreses in the presence of 1.0 % sodium dodecyl sulfate.

Analytical Methods. The concentration of purified cytochrome $\underline{b}_5$ was determined from the absorbance of Soret peak of the oxidized form assuming a millimolar extinction coefficient of 117 at 413 nm (9). The absorbance increment between 424 and 409 nm (185 $mM^{-1}cm^{-1}$) in the NADH-reduced minus oxidized difference spectrum was employed to calculate the cytochrome $\underline{b}_5$ content in membrane preparations (10). NADH-cytochrome $\underline{b}_5$ reductase was assayed by measuring its NADH-ferricyanide reductase activity (11). NADH-cytochrome $\underline{c}$ reductase activity, which is catalyzed by a cooperative action of NADH-cytochrome $\underline{b}_5$ reductase and cytochrome $\underline{b}_5$ (1), was determined as described by Dallner (12). Protein was estimated by the method of Lowry et al. (13), using bovine serum albumin as the standard.

Binding of Cytochrome $\underline{b}_5$ to Microsomes. Binding of cytochrome $\underline{b}_5$ preparations to microsomes was studied as follows. Usually, 0.3 ml of a mixture containing microsomes (11 mg of protein per ml), 50 mM phosphate buffer (pH 7.5), and a desired amount of d-$\underline{b}_5$ or t-$\underline{b}_5$ was incubated for 15 min at 37°C or 0°C. The incubation was stopped by diluting the mixture with at least 10 volumes of cold 0.1 M phosphate buffer (pH 7.5). The diluted mixture

was immediately centrifuged at 105,000 x g for 60 min. The pellet was resuspended in 4 ml of the same buffer and analyzed for protein, cytochrome $\underline{b}_5$, and enzymatic activities.

RESULTS

As shown in Fig. 1, considerable amounts of d-$\underline{b}_5$ could bind to rabbit liver microsemes when both were mixed and incubated for 15 min. Fig. 1 also shows that the incorporation occurred more efficiently at 37°C than at 0°C, and that the amount of d-$\underline{b}_5$ incorporated was dependent on the concentration of d-$\underline{b}_5$ added in the incubation mixture. Fig. 2 shows a double reciprocal plot of the microsomal content of cytochrome $\underline{b}_5$ after incubation at 37°C for 15 min against the concentration of d-$\underline{b}_5$ added in the incubation mixture. (As described below, incubation for 15 min under these conditions was sufficient for practically complete incorporation of d-$\underline{b}_5$ into microsomes.) The linearity of this plot suggests that the incorporation of d-$\underline{b}_5$ into microsomes becomes saturated when the concentration of external d-$\underline{b}_5$ is increased. It can be calculated from Fig. 2 that at the saturation the cytochrome $\underline{b}_5$ content in the incubated microsomes can be as 6.8-fold as high as that in the original microsomes. In addition to that Fig. 2 also suggestes that reversible step(s) are involved in this binding reaction. Since the assay for bound cytochrome $\underline{b}_5$ was conducted by reducing it with NADH *via* microsomal NADH-cytochrome $\underline{b}_5$ reductase, it was certain that the d-$\underline{b}_5$ detected was in a functional interaction with the reductase.

Fig. 3 shows that the time courses of d-$\underline{b}_5$ binding to rabbit liver microsomes at 37°C and 0°C. The binding was a relatively slow process, especially at the low temperature. At 37°C the incorporation was practically completed after 15-20 min of incubation, but even after 15 hr the incorporation at 0°C continued. The apparently higher content of cytochrome $\underline{b}_5$ in microsomes at

time zero than that in the original micosomes was perhaps due to the binding during centrifugal recovery of the microsomes.

Repeated washing with 0.3 M KCl or 10 mM EDTA in 0.1 M phosphate buffer (pH 7.5) could not remove cytochrome $\underline{b}_5$ that had been bound at 37°C to rabbit liver microsomes (Fig. 4). The same result was obtained for the d-$\underline{b}_5$ bound at 0°C. This excludes the possibility that the binding of d-$\underline{b}_5$ is due to electrostatic forces or involves divalent cations such as Ca^{2+} and Mg^{2+}.

If the hydrophobic moiety of d-$\underline{b}_5$ takes part in the binding of the protein to micosomes, t-$\underline{b}_5$ which lacks the hydrophobic moiety will not be able to bind to microsomes. As expected, it is indicated in Table 1 that t-$\underline{b}_5$ was incapable of binding to rabbit liver microsomes. It is also shown that microsomal NADH-ferricyanide reductase activity did not undergo any significant change by the binding of d-$\underline{b}_5$, and that obvious increased in microsomal NADH-cytochrome $\underline{c}$ reductase activity was caused by d-$\underline{b}_5$ binding, indicating the occurrence of a functional interaction of the incorporated d-$\underline{b}_5$ with endogenous NADH-cytochrome $\underline{b}_5$ reductase.

DISCUSSION

In this study, it is shown that conciderable amounts of d-$\underline{b}_5$ added externally can be incorporated into liver microsomes. This binding of d-$\underline{b}_5$ to microsomes is not mediated by electrostatic forces but is maintained by a hydrophobic interaction between the membrane matrix and the hydrophobic moiety of d-$\underline{b}_5$, because repeated washing with 0.3 M KCl or 10 mM EDTA could not liberate the once incorporated d-$\underline{b}_5$ from microsomes, and t-$\underline{b}_5$ which lacks the hydrophobic moiety was not able to bind to microsomes.

The ability of incorporated d-$\underline{b}_5$ to interact with NADH-cytochrome $\underline{b}_5$ reductase suggests that the d-$\underline{b}_5$ binding observed is of physiological significance. The d-$\underline{b}_5$ incorporated into microsomes must exist in essentially the same state as endogenous cytochrome $\underline{b}_5$ with regard to the enzymatic

functions in microsomes. Therefore it will be impossible to distinguish endogeneous cytochrome $\underline{b}_5$ from incorporated cytochrome $\underline{b}_5$.

Though the detailed mechanism of the process of d-$\underline{b}_5$ binding to microsomes is not yet clear, it is a relatively slow reaction even at 37°C. This indicates that the binding of d-$\underline{b}_5$ is not due to simple absorption. As shown in Fig. 2, the binding is very likely to invole a certain reversible process(es). It is also concievable that a state of equilibrium is reached after the incubation at 37°C for 15-20 min, for practically no more cytochrome $\underline{b}_5$ was incorporated into microsomes in spite of a significant amount of cytochrome $\underline{b}_5$ remaining in the solution. The conversion of cytochrome $\underline{b}_5$ from the state of micelles to that inserted into the membrane may require a certain amount of activation energy. The fact that the shift-up of the incubation temperature increases the rate of the reaction is compatible with this working hypothesis but the possibility that a change in the internal structure of the microsomal membrane itself at higher temperatures is involved should not be overlooked.

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FIG. 1 Binding of d-$\underline{b}_5$ to rabbit liver microsomes at 0°C and 37°C. The incubation was conducted under the conditions described in "EXPERIMENTAL PROCEDURE" using the indicated concentration of d-$\underline{b}_5$. The incubation was stopped by diluting the mixture 21-fold.

FIG. 2 A double reciprocal plot of the microsomal content of the bound d-$\underline{b}_5$ against the concentration of d-$\underline{b}_5$ in the incubation mixture after incubation at 37°C for 15 min. Data in Fig. 1 were employed. Endogeneous cytochrome $\underline{b}_5$ (0.95 nmole per mg of protein) was subtracted from the microsomal content of the cytochrome.

FIG. 3 Time courses of d-$\underline{b}_5$ binding to rabbit liver microsomes at 0°C and 37°C. 0.15 ml of 160 uM d-$\underline{b}_5$ and 0.15 ml of microsomes (22 mg of protein per ml), both in 50 mM phosphate buffer and preincubated at 0°C or 37°C for 1 min, were mixed and the mixture was incubated at indicated temperature for 2,5,10, or 15 min. The incubation was stopped by adding 8 ml of cold 0.1M phosphate buffer, pH 7.5. For the time zero control, the preincubated d-$\underline{b}_5$ and microsomes (both 0.15 ml) were separately diluted with 4 ml of the buffer and then mixed.

FIG. 4 Effect of washing on microsomal bound d-$\underline{b}_5$. Binding of d-$\underline{b}_5$ to rabbit liver microsomes was conducted at 37°C under the conditions in "EXPERIMENTAL PROCEDURE" using 50 uM d-$\underline{b}_5$. After the incubation, the mixture was diluted 10-fold with 0.1 M phosphate buffer, pH 7.5, and centrifuged at 105,000 x g for 60 min. The cytochrome $\underline{b}_5$ content in the resultant pellet was taken as the standard. The pollet was resuspended in 0.1 M phosphate buffer, pH 7.5, containing either 0.3 M KCl or 10 mM EDTA and centrifuged to effect the first washing. The second and third washings were performed in the same way.

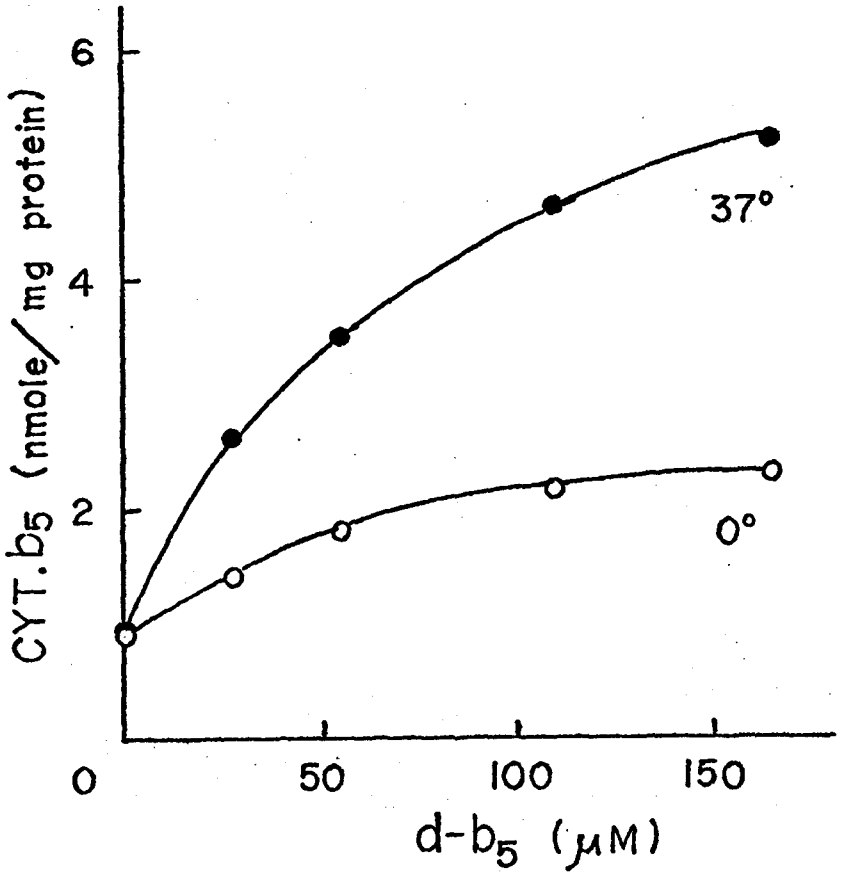

Fig. 1

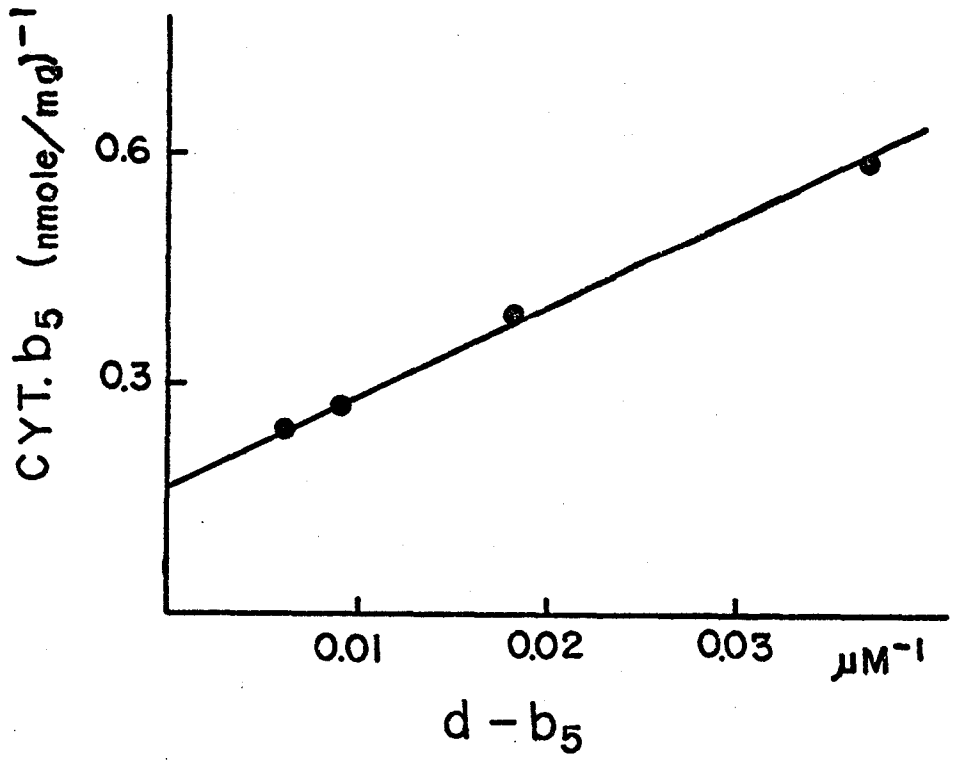

Fig. 2

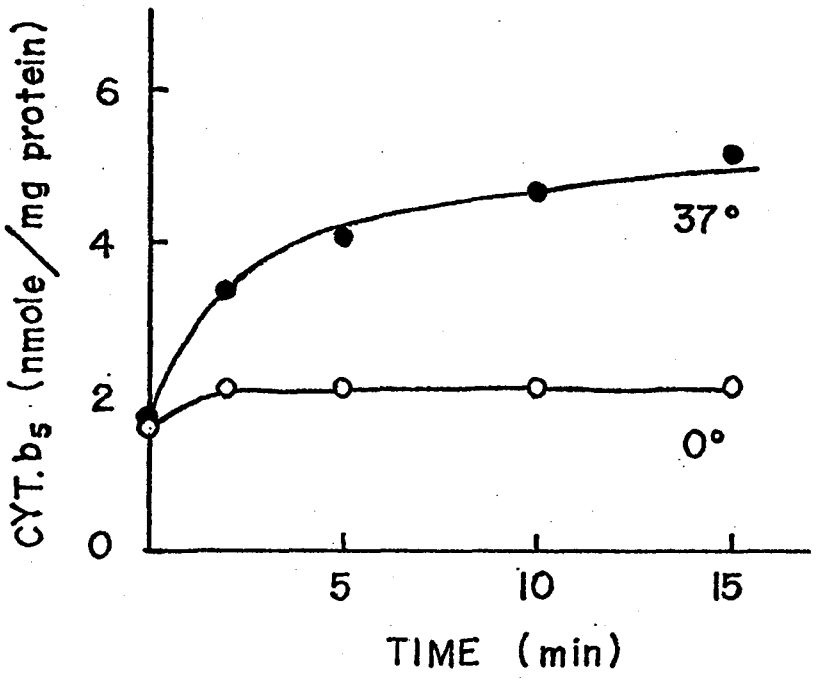

Fig. 3

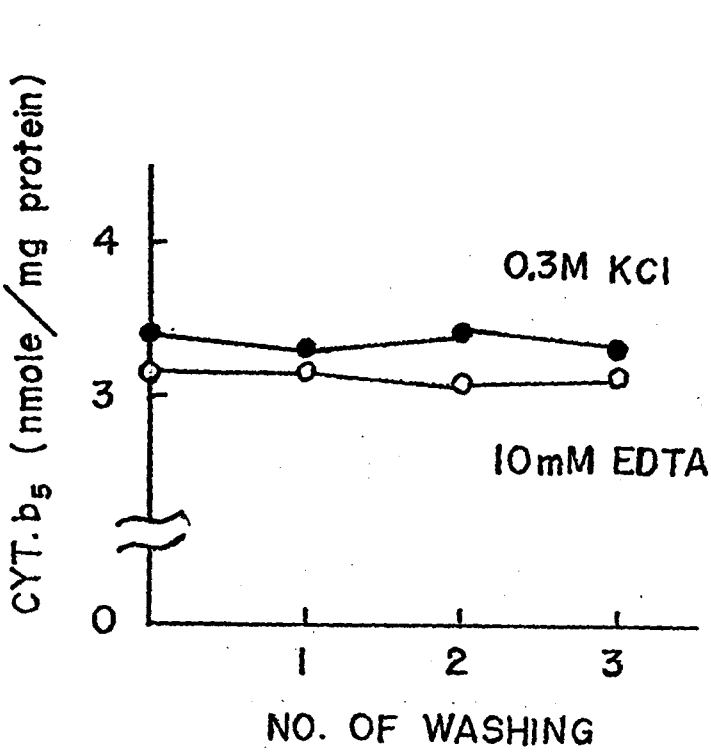

Fig. 4

TABLE I. LACK OF t-b_5 BINDING TO RABBIT LIVER MICROSOMES

Incubation Conditions	Specific Activity in Incubated Microsomes		
	Cytochrome b_5 (nmole/mg)	NADH-Cyt. c Reductase (unit/mg)	NADH-Ferricyanide Reductase (unit/mg)
0°C, no addition	0.90	0.58	2.83
0°C, plus 55 uM t-b_5	0.96	0.60	2.89
0°C, plus 55 uM d-b_5	1.81	1.18	2.86
37°C, no addition	0.95	0.63	3.00
37°C, plus 55 uM t-b_5	1.02	0.64	3.02
37°C, plus 55 uM d-b_5	3.50	1.49	2.60

The incubation was conducted under the standard conditions. The reaction was "stopped" by diluting the mixture 21-fold.

II. ASYMMETRIC BINDING OF CYTOCHROME b_5 TO HUMAN ERYTHROCYTE MEMBRANE

SUMMARY

The intact, amphipathic form of cytochrome b_5, purified from rabbit liver microsomes, could bind to unsealed human erythrocyte ghosts, but not to resealed ghosts, suggesting that the cytocheome could bind only to the inner (cytoplasmic) surface of the ghost membrane. This was further confirmed by the finding that the cytochrome could bind to closed, inside-out vesicles prepared from the ghosts. This asymmetric binding was not due to the exclusive localization of sialic acid and sugar chains on the outer surface of the ghost membrane, because the cytochrome was unable to bind to resealed ghosts even after enzymatic removal of these components. Although egg-yolk phosphatidylcholine liposomes could effectively bind the cytochrome, this binding capacity was progressively decreased as an increasing amount of cholesterol was included in the composition of the liposomes. The possibility is discussed that the asymmetric binding is due to preferential localization of cholesterol in the outer leaflet of the lipid bilayer that constitutes the ghost membrane.

INTRODUCTION

Previous studies have shown that both cytochrome b_5 (1,2) and NADH-cytochrome b_5 reductase (3,4), purified from liver microsomes after solubilization with detergents, are amphipathic proteins, each consisting of a hydrophilic moiety carrying the prosthetic group and a hydrophobic segment. Treatment of these proteins with proteases results in the cleavage of the molecules at the junction between the hydrophilic and hydrophobic parts, and the hydrophilic fragments thus produced still retain their respective catalytic activities (5). It has also been shown that the amphipathic form of these

proteins, but not their hydrophilic fragments, can bind effectively *in vitro* to liver microsomes, indicating that the hydrophobic segments of the proteins are responsible for their tight binding to the microsomal membrane (4, 6-8). However, the binding *in vitro* of these proteins is not restricted to microsomes; instead, they can also bind rather nonspecifically to a variety of natural and artificial lipid bilayer membranes such as those of liver mitochondria (4, 6) and phosphatidylcholine liposomes (4, 9-12). The only exception to this lack of specificity is the report by Strittmatter *et al.* (6) that cytochrome b_5 is unable to bind to human erythrocyte membrane, although Mihara and Sato (4) have reported efficient binding of NADH-cytochrome b_5 reductase to sonicated human erythrocyte ghosts.

This paper reports that the amphipathic form of cytochrome b_5 can bind only to the inner (cytoplasmic) surface of the erythrocyte ghost membrane, and presents evidence suggesting that asymmetric distribution of cholesterol in the ghost membrane is responsible for the asymmetric binding of cytochrome b_5.

EXPERIMENTAL PROCEDURE

Enzymes and Chemicals. The intact, amphipathic form of cytochrome b_5 (called "d-b_5") and its hydrophilic fragment (called "t-b_5") were purified from rabbit liver microsomes as described in Part I of this series. Trypsin, soybean trypsin inhibitor, and *Clostridium perfringens* neuraminidase were purchased from Sigma. Dextran T-10 and T-110 were obtained from Pharmacia. Egg-yolk phosphatidylcholine was purified by the method of Singleton *et al.* (13) and stored at -70°C under nitrogen gas. The other chemicals used were of the highest quality available.

Unsealed and Resealed Human Erythrocyte Ghosts. Outdated human blood in ACD solution was kindly supplied by Midori Juji Company, Ltd., Osaka. The erythrocytes obtained from the blood were washed three times with 5 mM

sodium phosphate buffer, pH 8.0, containing 0.15 M NaCl and then subjected to hemolysis in the buffer containing no NaCl. The resultant ghosts were washed repeatedly with 5 mM sodium phosphate buffer, pH 8.0, and finally suspended in 5 mM sodium phosphate buffer, pH 7.2, to a protein concentration of about 4 mg per ml. This suspension was used as the unsealed ghost preparation. The preparation of resealed ghosts was carried out essentially as described by Bodemann and Passow (14). Ten ml of the suspension of freshly prepared unsealed ghosts in 5 mM sodium phosphate buffer, pH 7.2 (about 4 mg of protein per ml), was slowly added with gentle stirring to 200 ml of 5 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.1 mM $MgSO_4$ (referred to as "isotonic buffer"). The mixture was incubated at 37°C for 60 min and then centrifuged at 20,000 x g for 10 min. The ghosts thus precipitsted were resuspended in the isotonic buffer to about 0.8 mg of protein per ml, and 15 ml of the suspension was layered over 15 ml of a dextran T-10 solution (1.6 g of dextran was dissolved in 50 ml of the isotonic buffer). This was then centrifuged at 22,500 rpm for 60 min in a Beckman SW 25.1 rotor. The resealed ghosts floating at the surface of the dextran layer were collected, washed with 10 vol. of the isotonic buffer, and suspended in the same buffer to a protein concentration of about 5 mg per ml.

Inside-Out Vesicles Derived from Erythrocyte Ghosts. Inside-out vesicles were prepared from erythrocyte ghosts essentially as described by Steck et al.(15). The pelleted unsealed ghosts obtained above were diluted with 40 vol. of 0.5 mM sodium phosphate buffer, pH 8.0, and the suspension was incubated at 0°C for 90 min. The ghosts recovered from the incubation mixture by centrifugation were converted to small vesicles by forcing them to pass through a hypodermic needle of gauge 27 at least 3 times. The homogenized ghosts were mixed with 2 vol. of 0.5 mM sodium phosphate buffer, pH 8.0. This suspension (5 ml) was layered over 5 ml of a dextran T-110 (8 g of dextran was dissolved

in 100 ml of 0.5 mM sodium phosphate buffer, pH 8.0 and centrifuged at 37,000 rpm for 60 min in a Beckman SW 41 rotor. The inside-out vesicles floating at the surface of the dextran layer were collected, washed with 0.5 mM sodium phosphate buffer, pH 8.0, and suspended in an appropriate buffer.

Phosphatidylcholine Liposomes Containing Cholesterol. A desired amount of cholesterol was dissolved in a chloroform solution containing a known amount of egg-yolk phosphatidylcholine. After removal of chloroform by evaporation, 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA was added to the residue to make the concentration of phospholipid phosphorus 10.6 mM, and suspension was mixed vigorously by means of a Thermo-Mixer. The preparation thus obtained contained large, multilamellar phosphatidylcholine liposomes containing known amount of cholesterol. These liposomes could be sedimented from the suspension by centrifugation at 105,000 x g for 30 min.

Treatments of Resealed Ghosts with Trypsin and Neuraminidase. Three ml of resealed ghosts suspended in the isotonic buffer (about 5 mg of protein per ml) was mixed with an equal volume of the isotonic buffer containing 136 ug of trypsin per ml, and the mixture was incubated at 37°C for 60 min. The ghosts were then precipitated and washed once with the isotonic buffer. The washed ghosts were resuspended in 5 ml of the isotonic buffer containing 124 ug of trypsin inhibitor per ml and the suspension was incubated at 37°C for 15 min. The trypsin-treated ghosts were again sedimented, washed with the isotonic buffer, and finally suspended in the same buffer. Neuraminidase treatment of the ghosts was carried out as follows. Resealed ghosts (about 15 mg of protein) suspended in 3 ml of the isotonic buffer were mixed first with 30 ml of 20 mM sodium phosphate buffer, pH 6.5, and then with 3 ml of the isotonic buffer containing 0.36 unit of neuraminidase. The mixture was incubated at 37°C for 60 min and then centrifuged. The pellet of neuraminidase-treated ghosts was washed once with the isotonic buffer and suspended in the

same buffer.

Binding of Cytochrome b_5 to Ghosts and Inside-Out Vesicles. Binding experiments with unsealed ghosts were conducted in 5 mM sodium phosphate buffer, pH 8.0, to prevent possible resealing during incubation, whereas the isotonic buffer was used for both resealed ghosts and inside-out vesicles. A mixture containing 60 uM cytochrome b_5 (either d-b_5 or t-b_5) and ghosts (either unsealed or resealed, 1.45 mg of protein) in 0.5 ml of the buffer was incubated at 37°C for 20 min. After the incubation, the mixture was diluted with 10 ml of the ice-cold buffer. The ghosts were sedimented from the diluted suspension by centrifugation, suspended in 5 ml of the buffer, and subjected to cytochrome b_5 and protein determinations. The binding of cytochrome b_5 to inside-out vesicles was similarly studied, except that the incubation mixture contained 55 uM cytochrome b_5 and inside-out vesicles (0.55 mg of protein) in a final volume of 0.5 ml.

Binding of Cytochrome b_5 to Liposomes. The incubation mixture contained, in a final volume of 0.5 ml, multilamellar phosphatidylcholine liposomes containing a desired amount of cholesterol (9.54 mM with respect to phospholipid phosphorus), 30 uM d-b_5, and 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA. After the incubation at 37°C for 30 min, the mixture was diluted with 4.5 ml of the EDTA-containing Tris-HCl buffer and centrifuged at 105,000 x g for 30 min. The precipitated liposomes and the supernate were then separately analyzed for cytochrome b_5.

Analytical and Assay Methods. Protein was determined by the Lowry method (16). Phospholipid phosphorus was estimated afer digestion by heating in conc. H_2SO_4 as described by Bartlett (17). Membrane-bound and neuraminidase-releleased sialic acid was measured by the method of Warren (18). Cytochrome b_5 in transparent samples was determined from the intensity of the oxidized Soret absorption peak at 413 nm, assuming a millimolar extinction coefficient of 117 (19). The cytochrome in membrane preparation was estimated from the

dithionite-reduced minus oxidized difference spectrum, assuming that the increment of millimolar extinction coefficient between 424 and 409 nm was 185 (20). Acetylcholine esterase and glyceraldehyde-3-phosphate dehydrogenase were assyed as described by Steck et al. (15).

RESULTS

Sealing Status and Membrane Sidedness of Ghosts and Vesicles. As a first step of this study, an experiment was carried out to check the sealing status of the human erythrocyte ghost preparations prepared as described above. For this purpose, glyceraldehyde-3-phosphate dehydrogenase (21) and acetylcholine esterase (22) were used as markers of the inner and outer surfaces of the ghost membrane, and it was assumed that the intact ghost membrane is impermeable to the substrates of these enzymes. It was also assumed that this permeability barrier is abolished completely by addition of Triton X-100.

As shown in Table I, glyceraldehyde-3-phosphate dehydrogenase activity of the resealed ghost preparation was mostly cryptic in the absence of Triton X-100, but became accessible to the exogenously added substrates on addition of the detergent. Acetylcholine esterase, on the other hand, was fully active regardless of the presence and absence of Triton X-100. These results indicated that most of the ghosts in this preparation were completely sealed. In the case of the unsealed ghost preparation, both marker enzymes were fully accessible to their substrates even in the absence of the detergent. It could be concluded that the ghosts in this preparation were actually unsealed, i.e. their membrane had holes permitting free entry of exogenously added substance. It is to be noted here that glyceraldehyde-3-phosphate dehydrogenase activity of the resealed preparation was much less than that of the unsealed preparation even after fully activated by the detergent. This was due to inactivation of the enzyme caused by exposure of the preparation to 37° C for 60 min

during the resealing process.

The results shown in Table II indicate that the membrane sidedness of the inside-out vesicles prepared from the ghosts was actually reversed. Thus, in contrast to the case of resealed ghosts, glyceraldehyde-3-phosphate dehydrogenase of the vesicles was fully active even in the absence of Triton X-100, whereas acetylcholine esterase became fully accessible to the substrate only after destruction of the membrane by the detergent. The reversed membrane sidedness was further confirmed by measuring the release of sialic acid, which is exclusively located on the outer surface of the ghost membrane (23), by neuraminidase treatment. In the absence of the detergent only a small amount of sialic acid was releasable from the vesicles by neuraminidase, whereas most of the sialic acid could be released in the presence of Triton X-100. These observations clearly indicated that most of the vesicles present in the preparation had reversed membrane sidedness and were completely sealed.

Selective Binding of Cytochrome $\underline{b}_5$ to Inner Surface of Ghost Membrane. Having confirmed the sealing status and membrane sidedness of the ghost and inside-out vesicle preparations, we then examined the binding of both the intact form of cytochrome $\underline{b}_5$ (d-$\underline{b}_5$) and its hydrophilic fragment (t-$\underline{b}_5$) to these membrane preparations. It was thus found that neither d-$\underline{b}_5$ nor t-$\underline{b}_5$ could bind to the resealed ghosts to significant extents (Table III), indicating that the outer surface of the ghost membrane was devoid of the cytochrome $\underline{b}_5$-binding capacity. It was, however, found that a considerable amount of d-$\underline{b}_5$ was bound by the unsealed ghosts in which the inner surface of the membrane was available for the added d-$\underline{b}_5$, suggesting that the binding of the cytochrome occurred at the inner surface. The possibility could, however, not be ruled out that the apparent binding was due to artifacts such as simple trapping of the cytochrome in the internal space of the ghosts caused by resealing during incubation. To avoid confusions arising from such artifacts,

the binding of the cytochrome to the inside-out vesicles was examined. As shown in Table IV, these vesicles could bind d-$\underline{b}_5$ effectively, providing firm evidence that d-$\underline{b}_5$ can in fact bind to the inner surface of the ghost membrane. In both the unsealed ghosts and inside-out vesicles, t-$\underline{b}_5$ bound to the membrane to much lesser extents than d-$\underline{b}_5$, as expected from the fact that t-$\underline{b}_5$ is a hydrophilic fragment of the intact cytochrome and lacks the hydrophobic, membrane-binding segment.

Effect of Removal of Sialic Acid and Sugar Chains from Ghosts. It has been reported that the sugar chains associated with proteins are located on the outer surface in the form of glycoproteins (24, 25), and the major glycoprotein of erythrocyte contains most of the cell surface sialic acid at the terminal position of the sugar chains (24, 26). It seemed, therefore, likely that the strongly negative charge of sialic acid is a factor preventing the binding of cytochrome $\underline{b}_5$ to the outer surface of the membrane, because cytochrome $\underline{b}_5$ is known to be an acidic protein (2) possessing a net negative charge at neutral pH. To test this possibility, about 80 % of the membrane-bound sialic acid was removed from the resealed ghosts by neuraminidase treatment. As shown in Table V, however, this treatment could not endow the ghosts with the cytochrome $\underline{b}_5$-binding capacity.

Another possibility is that the densely destributed sugar chains on the outer surface exert steric hindrance against the cytochrome binding. An attempt was , therefore, made to remove the sugar chains from the resealed ghosts by trypsin treatment. It was hoped that this treatment would cleave part of the glycoprotein molecules together with attached sugar chains from the outer surface of the membrane. As seen in Table V, this treatment decreased the sialic acid content of the ghosts from 31.3 to 12.7 ug per mg of protein. This result indicates that the trypsin treatment had removed considerably more than 60 % of the sugar chains, when the fact that trypsin

also removed a considerable portion of membrane protein is taken into consideration. At any rate, practically no binding of cytochrome $\underline{b}_5$ could be observed to the trypsin-treated ghosts. It was, therefore, unlikely that both the negative charge of sialic acid and densely distributed sugar chains act as barriers against the cytochrome binding.

Effect of Cholesterol on Cytochrome $\underline{b}_5$ Binding to Liposomes. In contrast to intracellular membranes such as microsomes, plasma membranes of animal cells including human erythrocyte are characterized by high contents of cholesterol. It is, therefore, likely that cholesterol has something to do with the asymmetric binding of cytochrome $\underline{b}_5$ to the erythrocyte ghost membrane, To obtain a clue to the possible role of cholesterol in regulation of cytochrome $\underline{b}_5$ binding to the ghost membrane, we took advantage of the fact that phosphatidylcholine liposomes can bind d-$\underline{b}_5$ effectively (9-12). Thus, multilamellar liposomes composed of egg-yolk phosphatidylcholine and cholesterol at various molar ratios were prepared, and the binding of d-$\underline{b}_5$ to these liposomes was examined. As shown in Fig. 1, the capacity of phosphatidylcholine liposomes to bind d-$\underline{b}_5$ was found to decrease progressively as the content of cholesterol in the liposomes increased. This result is a clear indication that the presence of cholesterol inhibits the binding of d-$\underline{b}_5$ to phospholipid bilayer membranes.

DISCUSSION

The results described above leave little doubt that the intact form of cytochrome $\underline{b}_5$ can bind only to the inner surface of human erythrocyte ghosts. It is evident that this asymmetric binding is a manifestation of the asymmetry of the organization of the ghost membrane. Based on the results obtained by several different techniques such as freez-fracture electron microscopy (27) and enzymatic and chemical labeling of proteins <u>in situ</u> (24, 28), it has been

reported that proteins of the erythrocyte membrane are mainly located in the inner layer and the outer surface is not saturated with proteins. If this is so, it is unlikely that the presence of proteins on the outer surface is the reason for the lack of binding of cytochrome $\underline{b}_5$. As already discussed, it is also inconceivable that the negative charge of sialic acid and dense distribution of sugar chains of glycoproteins act as barriers against cytochrome $\underline{b}_5$ binding to the outer surface of the ghost membrane, although there is still the possibility that sugar chains attached to membrane glycolipids, which cannot be removed by trypsin treatment, is responsible for the lack of binding capacity.

It seems most likely from these considerations that the asymmetric binding is mainly caused by asymmetric distribution or organization of lipid components within the erythrocyte membrane. In this connection, it should be mentioned that several investigators have presented evidence that phosphatidylcholine and sphingomyelin are concentrated in the outer layer of the erythrocyte membrane (29-31). The preferential localization of phosphatidylcholine in the outer layer, however, does not seem to be responsible for the phenomenon in question, because phosphatidylcholine liposomes can bind cytochrome $\underline{b}_5$ effectively as reported in this study and by other workers (9-12). Although the binding of cytochrome $\underline{b}_5$ to sphingomyelin liposomes has not yet been studied, it should be pointed out that sphingomyelin is similar to phosphatidylcholine in that both contain phosphorylcholine moiety in their molecules. It is also known that the ratio of phosphatidylcholine to sphingomyelin in the erythrocyte membrane varies greatly depending on the species of animal (29, 32). For instance, in sheep erythrocytes sphingomyelin is the major phospholipid which is present in a much greater quantity than phosphatidylcholine (32), and in this case sphingomyelin is believed to substitute phosphatidylcholine functionally. It is, therefore, not very likely that this choline-containing molecule inhibits

the cytochrome binding.

One of the characteristic features of plasma membranes of animal cells including human erythrocytes is their high contents of cholesterol and, therefore, this lipid component has to be considered as a candidate causing the asymmetric binding of cytochrome $\underline{b}_5$. As a matter of fact, it has been clearly shown in the present study that the cytochrome binding to egg-yolk phosphatidylcholine liposomes is inhibited by inclusion of cholesterol in the composition of the liposomes. It is, therefore, not unreasonable to assume that cholesterol is also exerting a similar effect in the erythrocyte ghost membrane. If this is the case, one has to postulate that the content of cholesterol in the outer layer of the ghost membrane must be much higher than that in the inner layer in order to explain the observed asymmetric binding of cytochrome $\underline{b}_5$. Another possibility may be to assume that there is a significant difference in the mode of orientation, but not the content, of cholesterol between the two layers. However, little is as yet known of the arrangement and location of cholesterol in the membrane. Recently, Fisher (33) has developed a method to obtain the outer layer of erythrocyte membrane based on the freeze-fracture technique. This method seems to be useful to prove the asymmetric localization of cholesterol in the two layers of the erythrocyte membrane. Although further work is surely needed to reach a decisive conclusion, it may be tentatively concluded that the preferential localization of cholesterol in the outer leaflet of the lipid bilayer of the erythrocyte membrane is mainly responsible for the asymmetric binding of cytochrome $\underline{b}_5$.

At present, nothing is known of the mechanism by which cholesterol inhibits the binding of cytochrome $\underline{b}_5$ to phospholipid membranes. Recent NMR studies have, however, provided evidence that cholesterol in phosphatidylcholine-cholesterol liposomes forms a complex with phosphatidylcholine at

a molar ratio of 1:1 (34). If this is so, then it is likely that cholesterol inhibits the binding by competing with cytochrome $\underline{b}_5$ for phosphatidylcholine which also acts as the cytochrome binding site (9-12). Cholesterol has also been shown to decrease the fluidity of various membranes (35-37). This effect may also be involved in the cholesterol-induced inhibition of the cytochrome binding.

In the present study we have shown that cytochrome $\underline{b}_5$, an amphipathic membrane protein, binds to the inner, but not outer, surface of human erythrocyte ghost membrane. It appears likely that this property is not restricted to cytochrome $\underline{b}_5$, but is shared by many other amphipathic proteins such as NADH-cytochrome $\underline{b}_5$ reductase (3, 4, 8). In this connection, the observation by Mihara and Sato (4) that the amphipathic form of the reductase could bind effectively to sonicated human erythrocyte ghosts deserves comment, since we have also observed the efficient binding of d-$\underline{b}_5$ to the same preparation. A preliminary experiment indicated that sonication of ghosts produces unsealed vesicles or fragments of the membrane, which agree with reports by other workers (38).

Finally, the asymmetric binding of cytochrome $\underline{b}_5$ reported in this paper and the possible involvement of cholesterol in this phenomenon may be regarded as representing one of the mechanisms by which various membrane proteins are distributed to their specific intracellular locations *in vivo*.

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TABLE I. Sealing Status and Membrane Sidedness of Ghosts.

TABLE II. Sealing Status and Membrane Sidedness of Inside-Out Vesicles.

TABLE III. Slective Binding of Cytochrome $\underline{b}_5$ to Unsealed Ghosts.

TABLE IV. Binding of Cytochrome $\underline{b}_5$ to Inside-Out Vesicles.

TABLE V. Lack of Binding of Cytochrome $\underline{b}_5$ to Ghosts Treated with Neuraminidase and Trypsin. The sialic acid content of untreated resealed ghosts was assayed as a control to estimate the effects of the enzymes used.

FIG. I Effect of Cholesterol on Cytochrome $\underline{b}_5$ Binding to Liposomes

markers	ghosts	detergent	activity (U/mg)	% accessibility
glyceraldehyde-3-P dehydrogenase	unsealed	+	1.98	98
		-	1.94	
	resealed	+	0.134	14
		-	0.019	
acetylcholine esterase	unsealed	+	1.51	103
		-	1.55	
	resealed	+	1.42	106
		-	1.50	

Table 1

markers	detergent	activity (U/mg)	% accessibility
glyceraldehyde-3-P dehydrogenase	+	2.37	95
	-	2.25	
acetylcholine esterase	+	1.85	24
	-	0.44	
sialic acid releasing	+	118*	14
	-	16.8	

* sialic acid released (nmoles/mg protein)

Table 2

ghosts	cytochrome	bound cytochrome (nmoles / mg protein)
resealed	+ d-$\underline{b}_5$	< 0.3
	+ t-$\underline{b}_5$	< 0.3
unsealed	+ d-$\underline{b}_5$	2.42
	+ t-$\underline{b}_5$	0.81

Table 3

cytochrome	bound cytochrome (nmoles/mg protein)
+ d-$\underline{b}_5$	1.98
+ t-$\underline{b}_5$	0.52

Table 4

ghosts	cytochrome	bound cytochrome (nmoles/mg protein)	sialic acid (μg/mg)
neuraminidase-treated	+ d-b_5	0.33	5.9
	+ t-b_5	0.49	
trypsin-treated	+ d-b_5	$<$0.3	12.7
	+ t-b_5	0.46	
resealed	———	———	31.3

Table 5

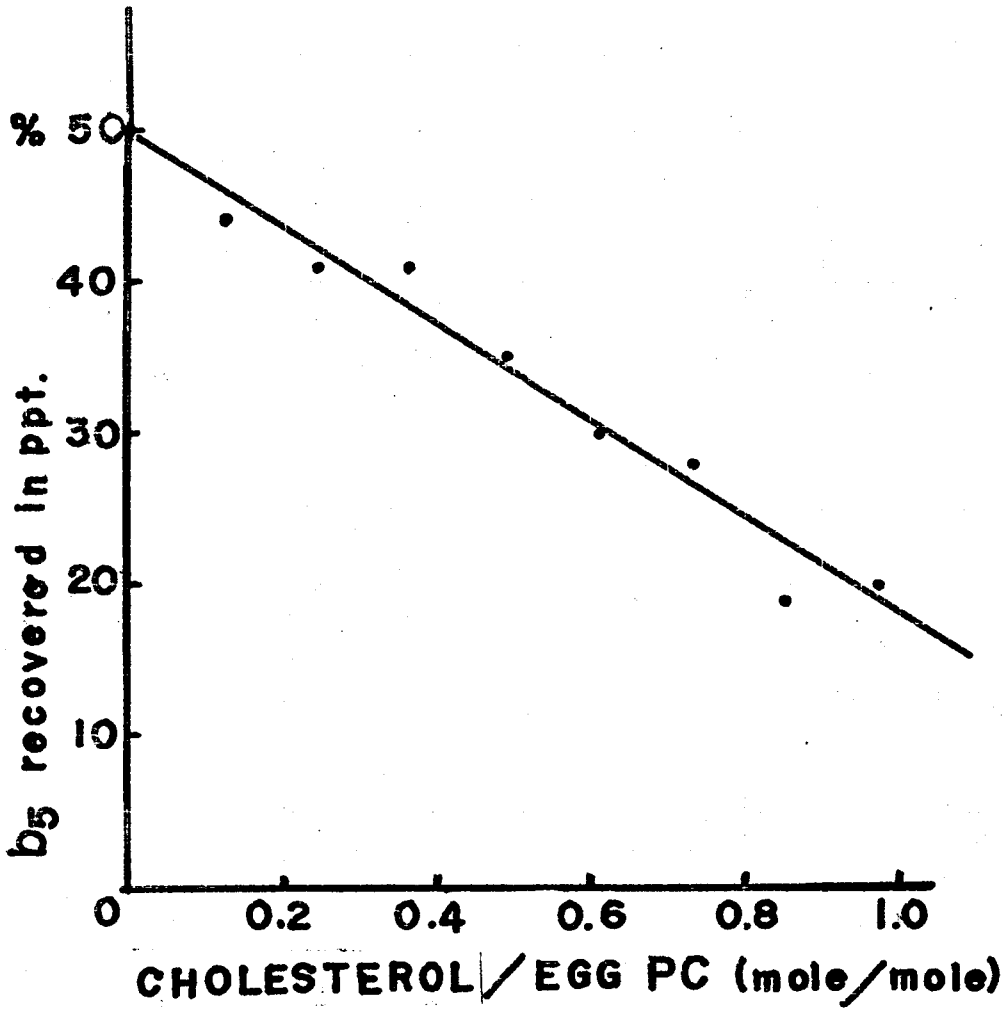
% 50
40
30
20
10
0
b_5 recovered in ppt.
0.2
0.4
0.6
0.8
1.0
CHOLESTEROL / EGG PC (mole/mole)

Fig. 1

III. FORMATION AND FUSION OF PHOSPHATIDYLCHOLINE-CYTOCHROME b_5 COMPLEXES

SUMMARY

Incubation of the intact form of cytochrome b_5 with single-walled liposomes of egg-yolk phosphatidylcholine resulted in the formation of at least four discrete species of phosphatidylcholine-cytochrome b_5 complexes having buoyant densities of 1.17, 1.15, 1.12 and 1.08. The species and quantities of the complexes formed varied depending upon the incubation conditions. Evidence was obtained to suggest that the initial event in the incubation is the formation of the heaviest complex in which the molar ratio of phosphatidylchomine to cytochrome b_5 is about 10 and that this complex undergoes fusion with liposomes containing no cytochrome b_5, leading to the formation of the complexes having lower buoyant densities with concomitant expansion of the size of the liposomal vesicles. The cytochrome b_5 molecules in the complexes were exclusively located on the outer surface of the vesicles. By using radioiodinated cytochrome b_5 preparations, it was found that cytochrome b_5 added exogenously could exchange with the cytochrome bound to liposomes.

INTRODUCTION

The intact form of cytochrome b_5 has been shown to have a structure consisting of two discrete domains which are conformationally independent to each other (1,2). One domain is hydrophilic and contains the heme whereas the other is hydrophobic and is involved in the binding of the cytochrome to the microsomal membrane (3, 4). In Part I of this series, we showed that the intact form of purified cytochrome b_5, but not the hydrophilic fragment of the hemoprotein, can effectively bind *in vitro* to liver microsomes. Taking advantage of this binding capacity of intact cytochrome b_5, Rogers and Strittmatter (5) have studied the behavior of the cytochrome on the microsomal membrane and reported that the protein is randomly distributed and capable of free trans-

lational movement on the surface of the membrane. However, the molecular organization of microsomal membrane is too complex to elucidate the behavior of cytochrome $\underline{b}_5$ on lipid bilayer membranes. A suitable system for this purpose is liposomes consisting of a chemically defined phospholipid or a mixture of phospholipids. In fact, Sullivan and Holloway (6) and other workers (7, 8, 9) have shown that cyrochrome $\underline{b}_5$ can bind effectively to phosphatidylcholine liposomes, but characterzation of the resultant lipid-cytochrome $\underline{b}_5$ complexes has so far been done only insufficiently. The purpose of this study was to establish a simple system for clarifying the interaction between cytochrome $\underline{b}_5$ and lipid bilayer membranes. In this paper, we describe improved methods for preparation and analysis of the complexes formed from single-walled egg-yolk phosphatidylcholine liposomes and cytochrome $\underline{b}_5$. In addition, it is reported that the liposomes to which cytochrome $\underline{b}_5$ has been bound undergo membrane fusion with other liposomes and that exogenously added cytochrome $\underline{b}_5$ can exchange with the cytochrome in the phosphatidylcholine-cytochrome $\underline{b}_5$ complexes.

EXPERIMENTAL PROCEDURE

Enzymes and Chemicals. The intact form of cytochrome $\underline{b}_5$ (d-$\underline{b}_5$) was purified from rabbit liver microsomes as described in Part I. The method of Takesue and Omura (10) was used to purify lysosome-solubilized NADH-cyrochrome $\underline{b}_5$ reductase from rabbit liver microsomes. The reductase thus purified lacks the hydrophobic region and thus is unable to bind to membranes (11, 12). Phosphatidylcholine was isolated and purified from egg yolks by the method of Singleton et al. (13). The purity of the phospholipid preparation was confirmed by thin-layer chromatography and stored at -70°C under nitrogen gas. ^{125}I- and ^{131}I-labeled sodium iodide were purchased from the Japan Isotope Association. The other chemicals employed were of the highest quality available.

Preparation of Single-Walled Liposomes. Single-walled liposomes of egg-yolk phosphatidylcholine were prepared by a modification of the method of Huang (14). About 0.3 g of phosphatidylcholine was dispersed in 10 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA with the aid of a Thermo-Mixer. The suspension placed in an ice bath was sonicated under a stream of nitrogen gas for 30 min at the full power of a Branson sonifier equipped with a micro tip. The sonicated suspension was centrifuged at 105,000 x g for 60 min, and the supernate was filtered through a Toyo TM-5 membrane filter. The filtrate was then subjected to chromatography on a Sephafose 4B column (2.5 x 50 cm) using the same buffer as eluant. Multi-layered lipsomes which were eluted at or the near void volume were discarded and the retarded fraction was collected and used as single-walled liposomes. If necessary, the liposome preparation thus obatined was concentrated in an Amicon ultrafiltration device equipped with a type PM-30 membrane. The preparation was homegeneous consisting of vesicles having a diameter of approximately 250 Å when checked by negative staining electron microscopy, as described by Huang (14).

Binding of Cytochrome b_5 to Liposomes. A mixture containing, in a final volume of 0.1 ml, 160 uM cytochrome b_5, a desired amount of single-walled liposomes of phosphatidylcholine, 50 mM Tris-HCl buffer, pH 8.0, and 1 mM EDTA was incubated at 0°C for 20-24 h or at 37°C for 5-30 min. After incubation, the mixture was layered over 5 ml of a linear concentration gradient of sucrose (5 to 46 % (w/v)) containing 50 mM Tris-HCl buffer, pH 8.0, and 1 mM EDTA, and the system was centrifuged at 2°C for 20 h in a Beckman SW 65 rotor at 50,000 rpm. After centrifugation, 2 or 3 drop fractions were collecfed from the bottom of the tube and analyzed for cytochrome b_5, phospholipid phosphorus, and sucrose concentration. For large scale experiments, 1 ml of the incubation mixture was layered over 30 ml of a linear sucrose density gradient (5 to 40 %) and centrifuged in a Beckman SW 25.1 rotor or a Hitachi RPS 25 rotor at 23,000 rpm for 24 or 68 h at 2°C. The collected fractions were dia-

lyzed against 50 mM Tris-HCl buffer, containing 1 mM EDTA.

Electron Microscopy. Phosphatidylcholine liposomes or phosphatidylcholine-cytochrome b_5 complexes were diluted withthe buffer to a suitable concentration and placed on a specimen mesh coated with collodione film. The sample was then stained with 2 % phosphotungstic acid, pH 7.2. After drying the mesh at room temperature, the specimen was observed in a Hitachi HU-12 electron microscope.

Radioiodination of cytochrome b_5. A solution containing 0.01 umole of cytochrome b_5 and 0.01 umole of cold NaI in 0.4 ml of 0.4 M sodium borate buffer, pH 9.0, containing 0.28 M NaCl was mixed with 0.2 mCi of $Na^{125}I$ or 1 mCi of $Na^{131}I$ in 0.2 ml of 0.005 N NaOH. Chloramine T (1 umole for ^{125}I and 2 umoles for ^{131}I experiments) in 0.2 ml of the buffer was then added and the mixture was incubated at room temperature for 10 to 20 min. The reaction was stopped by adding 1.3 or 2.6 umoles of Na_2SO_3 dissolved in 0.2 ml of the buffer. The iodinated protein was dialyzed extensively against 0.02 M sodium borate buffer, pH 9.0, containing 0.15 M NaCl. The specific radioactivities of the ^{125}I-labeled and ^{131}I-labeled cytochrome b_5 preparations were 0.016 and 0.036 mCi per nmole, respectively.

Analytical Methods. Cytochrome b_5 was determined as described in Part I. Phospholipid phosphorus was determined by the method of Bartlett (15) after digesting the sample by heating in conc. H_2SO_4. Sucrose concentraiion in fractions collected after density gradient centrifugation was determined by means of an Abbe refractometer. Radioactivity of ^{125}I and ^{131}I was measured in an Aloka Auto-Well Gamma System.

RESULTS

Formation of Phosphatidylcholine-Cytochrome b_5 Complexes. When a mixture containing single-walled phosphatidylcholine liposomes and cytochrome b_5 at a molar ratio of 37:1 was incubated for 5 min at 37°C and then subjected to sucrose density gradient centrifugation at 50,000 rpm for 20 h as described in

Experimental Procedure, two types of phospholipid-cytochrome $\underline{b}_5$ complexes were detected at densities of 1.12 and 1.08, as shown in Fig. 1(a). When the incubation time was extented to 30 min under otherwise the same conditions, another complex at a density of 1.15 was formed in addition to the above two complexes (Fig. 1(b)).

When the molar ratio of phosphatidylcholine to cytochrome $\underline{b}_5$ in the mixture was reduced to 9:1 and the incubation was performed for 5 min at 37°C, a major portion of cytochrome $\underline{b}_5$ was recovered in a complex locating at a density of 1.15, as shown in Fig. 1(c). This density was identical with that determined for the complex which appeared after 30 min incubation at the ratio of 37:1. In Fig. 1(c), a considerable amount of cytochrome $\underline{b}_5$ was also recovered in fractions having densities lower than 1.14. These fractions seemed to contain unbound cytochrome $\underline{b}_5$ together with some sorts of complexes, but their characterrization was not conducted because of the difficulty in separating these species. When the mixture at the molar ratio of 9:1 was incubated for 30 min at 37°C, a new species of complex was formed at a density as high as 1.17 in addition to the complex having a density of 1.15 (Fig. 1(d)). It was further found that this high density complex could also be formed upon incubation of a mixture at a molar ratio of 9:1 for 20 to 24 h at 0°C. As shown in Fig. 1(e), the cytochrome in this mixture after the incubation existed only in the form of complex having a density of 1.17 and in the unbound form which was floating in the upper region.

To examine if the centrifugal conditions employed in the above experiments (20 h at 50.000 rpm) were sufficient to reach the density equilibrium, the centrifugation was performed for 69 h with the other conditions unchanged. It was thus found that all the complexes described above, except that the one which had a density of 1.15 after centrifugation for 20 h, remained at the same positions, indicating that centrifugation for 20 h was sufficient to

determine the buoyant densities of the complexes formed. After centrifugation for 69 h, the complex having a density of 1.15 exhibited a minor shift to about 1.16 for unkown reasons. Cytochrome $\underline{b}_5$, when centrifuged for 69 h in the absence of liposomes, was recovered at a position corresponding to a density of approximately 1.17, instead of about 1.11 after centrifugation for 20 h. It was, therefore, evident that the unbound cytochrome had not reached the position of its own density after centrifugation for 20 h, because of its lower sedimentation coefficient than those of the complexes. At the molar ratio of 37:1 no significant amount of cytochrome $\underline{b}_5$ was detected at the density of 1.17 where the cytochrome was expected to locate when centrifuged for 69 h in the absence of liposomes. Under these conditions, therefore, most of the added cytochrome $\underline{b}_5$ had bound to the liposomes.

These observations suggested that the binding of cytochrome $\underline{b}_5$ to phosphatidylcholine liposomes may not be a random process but proceed in a discontinuous way. The complexes identified by the sucrose density gradient centrifugation method were classified into four types by their buoyant densities. We will refer these complexes to as Complexes I, II, III and IV which have buoyant densities of 1.17, 1.15, 1.12 and 1.08, respectively.

Preparation and Characterization of Various Complexes. To characterize further the aforementioned four types of complexes, they were isolated in large scale experiments. Complex I was formed by incubation of a mixture containing phosphatidylcholine liposomes and cytochrome $\underline{b}_5$ at a molar ratio of 9-10:1 at 0°C for 20 to 24 h and then separated from the unbound cytochrome by density gradient centrifugation at 23,000 rpm for 24 h. For preparation of the other complexes, a mixture of liposomes and cytochrome $\underline{b}_5$ at a molar ratio of 37:1 was incubated for 5 or 30 min at 37°C followed by centrifugation at 23,000 rpm for 68 h. Although the centrifugal force employed in these experiments was lower than that used above (50,000 rpm), the separation of complexes from one another was sufficient, as shown in Fig. 2. The complexes

thus isolated were dialyzed against the sucrose-free buffer and analyzed for phospholipid and cyrochrome $\underline{b}_5$.

In Table I are summarized the molar ratios of phospholipid to cytochrome $\underline{b}_5$ in the four types of complexes isolated above. The molar ratios were also calculated from the buoyant densities of the complexes and are included in Table I. This calculation was made based on an empirical rule pointed out by Parsons (16) that the buoyant density of a membrane preparation can be determined from the proportion of protein and lipid in that preparation. On this assumption, the following equation can be derived:

$$r = \frac{M_P (d_P - d_C)}{M_L (d_C - d_L)}$$

where r is the molar ratio of phosphatidylcholine to cytochrome $\underline{b}_5$ in the complex to be studied; M_P is the molecular weight of cytochrome $\underline{b}_5$, $\underline{i.e.}$ 16,700 (17); M_L is the average molecular weight of egg-yolk phosphatidylcholine, $\underline{i.e.}$ 770 (14); d_C is the buoyant density of the complex; d_P is the buoyant density of cytochrome $\underline{b}_5$; and d_L is the buoyant density of egg-yolk phosphatidylcholine. In calculation, we assumed that d_P is 1.27 (16) and d_L is 0.97, instead of 0.94 used by Parsons (16). As can be seen in Table I, the molar ratios thus calculated for the four complexes were in good agreement with those determined directly by chemical analysis, except for Complex IV, which contained much more phosphatidylcholine than expected from calculation. It was likely that this discrepancy was due to contamination of the complex by cytochrome $\underline{b}_5$-free liposomes.

Fig. 3 shows the electron micrographs of negatively stained preparations of the four types of complexes. In the preparations of Complexes I, II and III, abundant vesicllar profiles having a diameter ranging from 250 to 500 Å were observed, whereas the preparation of Complex IV contained larger vesicles whose diameters were up to 700 Å. Thus, the diameters of most vesicle's seen

in all the preparations were larger than that of the original phosphatidylcholine liposomes which have been reported to have diameter of about 250 Å (14). When the preparations were incubated for 2 days at 0-4 °C and then subjected to negative staining electron microscopy, the number of larger vesicles was increased and there appeared larger vesicles which had not been found before the incubation, as illustrated in Fig.3 (e) and (d) for Complexes I and III. The reason for this phenomenon will be discussed in a later section.

Localization of Cytochrome b_5 in Complexes. Since the lipid bilayer of liposomes is impermeable to macromoleculas such as cytochrome b_5, it is likely that the cytochrome in the complexes is located exclusively on the outer surface of the liposome membrane. To check this possibility, an attempt was made to see if the cytochrome b_5 bound to the complexes was reducible by exogenously added NADH- and lysosome-solubilized NADH-cytochrome b_5 reductase. Under these conditions, it could be expected that only the cytochrome bound to the outer surface was reducible, because the reductase preparation used was capable of neither penetrating the bilayer nor binding to the membrane. As shown in Table II, all the cyrochrome in the complexes was reducible by the exogenously added NADH and the reductase; the amount of cytochrome b_5 thus reduced was the same as that reduced by dithionite which was added to the system after destruction of liposome membrane with Triton X-100. The localization of cytochrome b_5 on the outer surface of the liposome membrane could blso be confirmed by electron microscopic observation of the negatively stained complexes. Although the naked liposomes have a smooth surface, small knob-like structures having a diameter of about 30 Å are clearly detected on the surface of the complexes, as shown in Fig. 4. The size of these structures is in good agreement with that of the hydrophilic heme-containing fragment of the cytochrome molecule, which has been shown to be ellipsoidal in shape and 25 x 25 x 32 Å in dimension (18). It is thus highly likely that the cytochrome in the complexes is attached to liposomes by inserting its hydrophobic tail into the

lipid bilayer and exposing its hydrophilic moiety to the surrounding aqueous phase.

Fusion of Cytochrome b_5-Containing Liposomes. As described above, incubation of phosphatidylcholine liposomes with cytochrome b_5 results in the formation of discrete types of complexes having unique buoyant densities. This fact, together with the observation that the complexes having lower densities tended to possess larger diameters, suggest the possibility that fusion of cytochrome b_5-containing liposomes with naked liposomes may be involved in the formation of the discrete types of complexes. A likely explanation for the observations is to assume the initial formation of liposomes which are completely or nearly completely saturated with cytochrome b_5 and the subsequent fusion of these saturated liposomes with other vesicles including those containing no cytochrome b_5. If this is the case, then it is expected that incubation of the complexes having high buoyant densities with naked liposomes will result in the appearance of new species of complexes having lower densities and concomitant expansion of the size of vesicles, To test this possibility experimentally, freshly prepared Complex I having a density of 1.17 was isolated and incubated with phosphatidylcholine liposomes, and the incubated mixture was analyzed by sucrose density gradient centrifugaion. As shown in Fig. 5, the formation of Complexes II, III and IV from the initially added Complex I was clearly detected, though the amounts and species of these complexes formed were dependent on the relative quantity of phosphatidylcholine liposomes added. Since the calculated molar ratios of phosphatidylcholine to cytochrome b_5 in Complexes II,III and IV are approximately 1.5-, 2- and 3-fold, respectively, higher than that of Complex I as shown in Table I, it could be suggested that Complex III, for instance, was formed by fusion of Complex I with an equivalent amount of liposomes containing no cytochrome b_5. The formation of Complexes II and IV could also be explained by fusion of Complex I with differnt quantities of liposomes. It was, therefore, concluded that

Complexes II, III and IV detected in the experiments shown in Fig. 1 were all derived from Complex I by fusion with liposomes containing no cytochrome b_5.

Exchange of Cytochrome b_5 between Complexes and Cytochrome b_5 Micelles. As suggested in Part I of this series, it is likely that the binding of cytochrome b_5 to microsomes and other membranes involves a reversible process. It seems that elucidation of this reversibility is of profound importance in studying the memhanism of cytochrome b_5 binding. The following expeiments were , therefore, performed to see if the binding process is actually reversible. Phosphatidylcholine liposomes and cytochrome b_5 iodinated with ^{131}I were incubated at 0°C for 24 h and ^{131}I-labeled Complex I was isolated therefrom. This preparation was then incubated at 37°C with cytochrome b_5 iodinated with ^{125}I, and subjected to sucrose density gradient centrifugation to separate the complex from the micelles of cytochrome b_5. Fig. 6 (a) shows that practically no liberation of ^{131}I-labeled cytochrome b_5 took place when the complex was incubated in the absence of added cytochrome b_5. As can be seen in Fig. 6 (b), on the other hand, significant amounts of ^{131}I radioactivity was recovered in the fraction corresponding to cytochrome b_5 micelles and at the same time comparable quantities of ^{125}I-labeled cytochrome b_5 sedimented with the complex, after the ^{131}I-labeled complex was incubated with ^{125}I-labeled cytochrome b_5. These results provided strong evidence that the cytochrome in the complex underwent an exchange reaction with the exogenoully added cytochrome. This in turn suggested that the binding of cytochrome b_5 to liposomes actually involves a reversible step(s) It was noticed that the iodinated cytochrome preparation possessed lower binding capacities than the unlabeled prepatation probably due to modifications of the molecule, and therefore quantitative discussions on the exchange reaction should be done with caution.

DISCUSSION

Although it has been repoted that cytochrome b_5 can effectively bind to phosphatidylcholine liposomes (6-9), little is as yet known of the mechanism of this interaction. It can be assumed that the cytochrome binds to liposomes in a random fashion. If this is the case, then it is expected that incubation of the cytochrome with liposomes should result in the formation of complexes having the sam lipid to cytochrome ratio. The results described above, however, indicate that the actual result observable is the formation of at least four discrete species of complex. i.e. Complexes I,II,III and IV, which differ from one another in buoyant density and the lipid to cytochrome ratio, although the species and quantity of the complexes formed vary depending on the incubation conditions, These results indicate clearly that a non-random mechanism is involved in the binding process.

The fact that the molar ratios of phosphatidylcholine to cytochrome b_5, determined both directly and by calculation from buoyant densites, for Complexes II, III and IV are 1.5-, 2- and 3- fold higher than that obtained for Complex I suggests that the former complexes are produced by fusion of Complex I with various numbers of liposomes which contain no cytochrome b_5. This possibility has actually been confirmed by the observation that incubation of Complex I with free liposomes results in the formation of Complexes II, III and IV (Fig. 5) and the increased size of veiicles in the lighter complexes. It seems, therefore, likely that the initial event in the bind ng process is the formation of Complex I in which cytochrome b_5 is densely attached to a liposome (about 10 moles of phosphatidylcholine per mole of cytochrome b_5). This unusual formation of the complex heavily loaded with the cytochrome as the intial product can be explained by assuming a mechanism in which cytochrome b_5 binds to a liposome in a cooporative way; in other words, the intial binding of the cytochrome to a liposome facilitates the subsequent binding of the cytochrome molecules to the same vesicle, probably because of alterations in

the properties of the membrane.

In any way, it is now highly likely that Comp ex I, in which the liposomal vesicle seems to be nearly saturated with cytochrome $\underline{b}_5$, then undergoes membrane fusion with free liposomes leading to the formation of Complexes II, III and IV. Since phosphatidylcholine liposomes themselves cannot form large vesicles even upon incubation at 37° (K. Mihara, personal communication), it is obvious that the fusion capacity is induced by the binding of cytochrome $\underline{b}_5$. Although no decisive explanation can yet been offered for this fusion capacity, it should be noted that the lipid-cytochrome $\underline{b}_5$ complexes possess a highly asymmetric structure in that all the cytochrome molecules in the complexes are located only on the outside surface of the vesicles, as evidenced by the reducibility of the cytochrome by ex genously added NADH and NADH-cytochrome $\underline{b}_5$ reductase and also by electron microscopic observations. It is conceivable that such an asymmetric locarion of the cytochrome molecules makes the liposomal membrane unstabele owing to the distortion between the outer and inner leaflets of the bilayer. Another possibility is that immobilization of lipid molecules by the bound cytochrome (19) is the cause of the membrane instabilization. Since the distortion is thought to be stronger in smaller vesicles having higher curvatures, a possible way to overcome the distortion may be to undergo fusion with other liposomes to form larger vesicles. If this is so, it is expected that the fusion continues untill the distortion is abolished. In this connection, it is of interest to note that the largest fusion product observed in this study has a diameter of about 700 Å. Assuming that the surface area of vesicles does not change before and after the fusion, it can be calculated that a fused liposome having a diameter of 750 Å is composed of approximately 9 liposomes having a diameter of 250 Å.

Another question to be answered is whether the binding of cytochrome $\underline{b}_5$ and the fusion are independent reactions or they are closely related to each other. The results reported in this paper suggest the independence of the

two processes. Thus, the experiment in which phosphatidylcholine and cytochrome b_5 were incubated at a molar ratio of 9:1 at 0°C and 37°C (Fig. 1) indicates that almost all the complexes formed at 0°C was identified as Complex I, whereas fused products such as Complex III were mainly formed at 37°C. This can be interpreted by assuming that the rate of fusion, as compared with that of binding, is considerably lower at 0°C than at 37°C. It is, however, to be noted that upon prolonged incubation fusion of Complex I does take place even at 0°C as revealed by electron microscopy. The fact that isolated Complex I undergoes fusion with free liposomes (Fig.5) also indicates the independence of the two processes.

Finally, the observation that an exchange reaction occurs between the cytochrome in Complex I and the micelles of cytochrome b_5 added is important for elucidation of the mechanism of cytochrome binding to lipid bilayer membrane, because it suggests that a reversible process is involved in the binding reaction. This exchange reaction may be generalized as that of an amphipathic protein between the micelles of the protein and mixed micelles of lipids or detergents containing the protein. In this connection, it is worth noting that the interaction of cytochrome b_5 with micelles of detergents such as Triton X-100 and deoxycholate has been studied in detail by Robinson *et al.* (7). Rogers and Strittmatter (20) havd reported that cytochrome b_5 which had bound *in vitro* to liver microsomes can be partially removed from the membrane by incubation with the intact form of NADH-cytochrome b_5 reductase. It is, however, not yet clear if their observation has anything to do with the exchange reaction described in this study, and furhter studies are requieed to obtain a conclusive solution of the mechanism of cytochrome b_5 binding to phospholipid bilayer membranes.

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FIG. 1 Formation of discrete tupes of complexes. The experiments were performed as described in Experimental Procedure. Binding of cytochrome $\underline{b}_5$ to phosphatidylcholine liposomes was conducted at a molar ratio of 37:1 at 37°C for 5(a) or 30 b) min. The mixture of a molar ratio of 9:1 was also incubated at 37°C for 5(c) or 30(d) min and at 0°C for 20 h(e).

FIG. 2 Preparation of the four types of complexes. Formation of Complexes I, II, III and IV was carried out as described in the text and the complexes were subjected to centr fugation at 23,000 rpm for 24(a) or 68(b) h. In the experiment shown in (a), cytochrome $\underline{b}_5$ was incubated at the concentration of 250 uM instead of 160 uM.

FIG. 3 Electron Microscopic profiles of various complexes. Freshly prepared Complexes I(a), II(b), III(c) and IV(d) were observed after staining with phosphotungstic acid as described in Experimental Procedure. Profiles of Complexes I and III after the incubation for 2 days at 0-4°C were shown in (e) and (f) respectively.

FIG. 4 Fine structure of the surface of complexes. Complex II was stained with phosphotungstic acid and observed at a magnification of x 70,000 as described in Experimental Procedure.

FIG. 5 Fusion of Complex I with phosphatidylcholine liposomes containing no cytochrome $\underline{b}_5$. A mixture of freshly prepared Complex I at a concen- of 0.92 mM for phosphatidylcholine and equivalent(a) or 2-fold(b) amount of phosphatidylcholine liposomes containing no cytochrome $\underline{b}_5$ was incubated at 37° for 5 min, then the mixture was subjected to linear sucrose density gradient (5-46 % (w/v) in 50 mM Tris-HCl buffer) centrifugation at 50,000 rpm for 66 h in a Beckman SW 65 rotor .

FIG. 6 Exchange of cytochrome $\underline{b}_5$ between complexes and cytochrome $\underline{b}_5$ micelles. A mixture of ^{131}I-labeled Complex I (87,000 cpm) containing 14.8 nmoles of cytochrome $\underline{b}_5$ in 1ml of 50 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA was incubated at 37°C for 30 min in the presence(b) and absence(a) of exogenously added ^{125}I-labeled cytochrome $\underline{b}_5$ (79.5 nmoles, 180,000 cpm). The mixture was, then, placed on a linear sucrose density gradient (5-40 %(w/v)) followed by centrifugation at 23,000 rpm for 24 h in a Beckman SW 25.1 rotor.

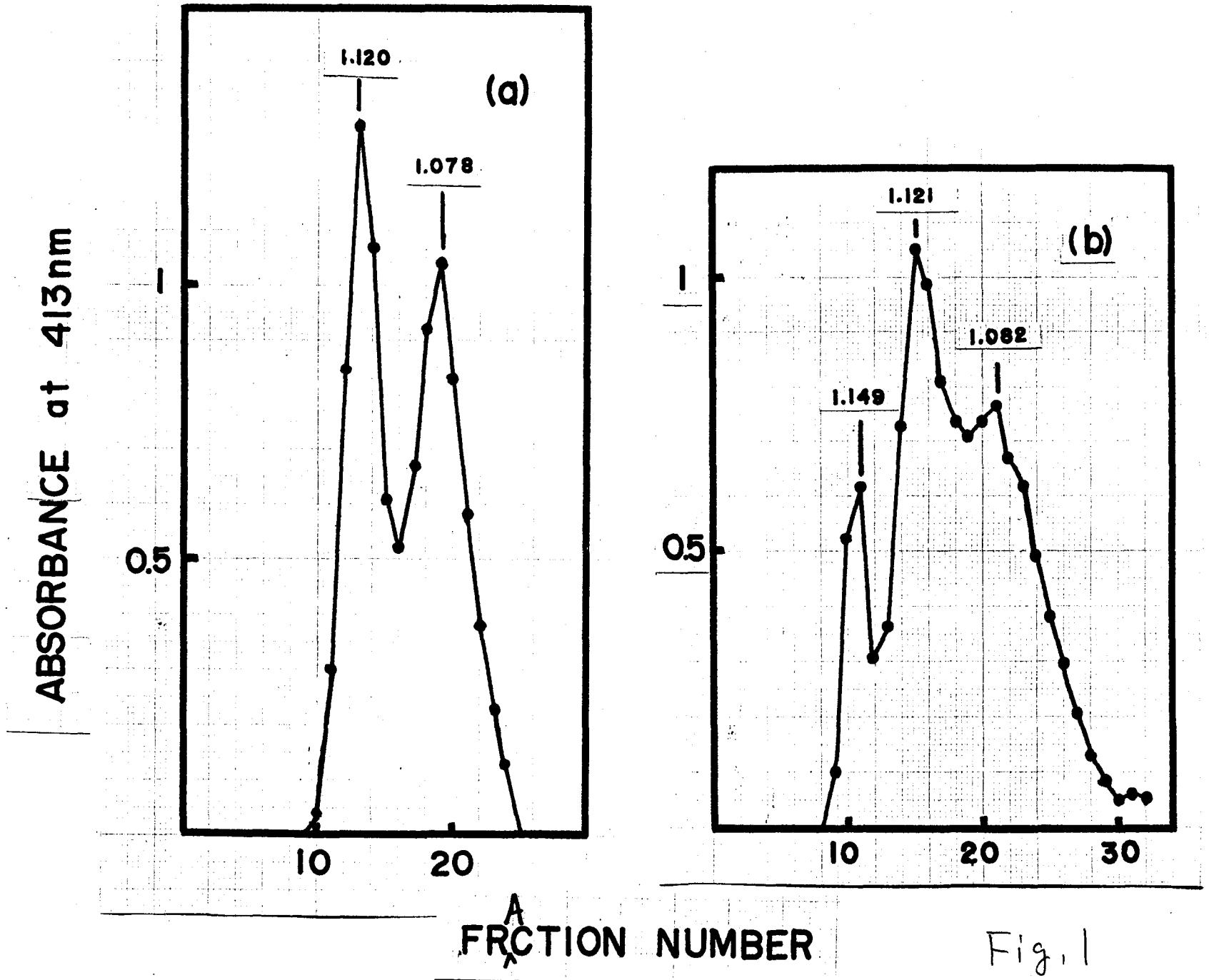

1.120
(a)
1.078
1.121
(b)
1.082
1.149
ABSORBANCE at 413nm
1
0.5
10
20
30
FRCTION NUMBER

Fig. 1

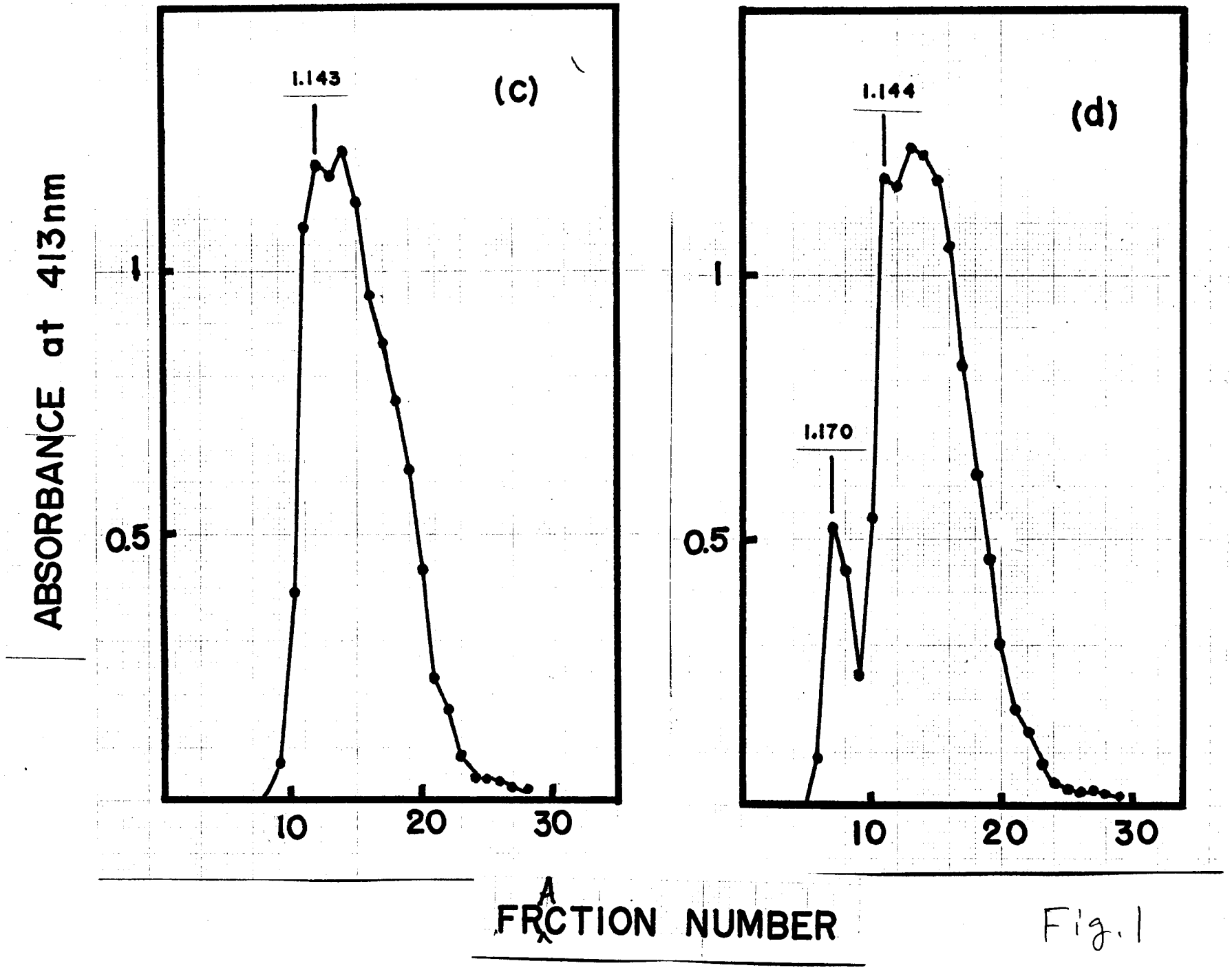
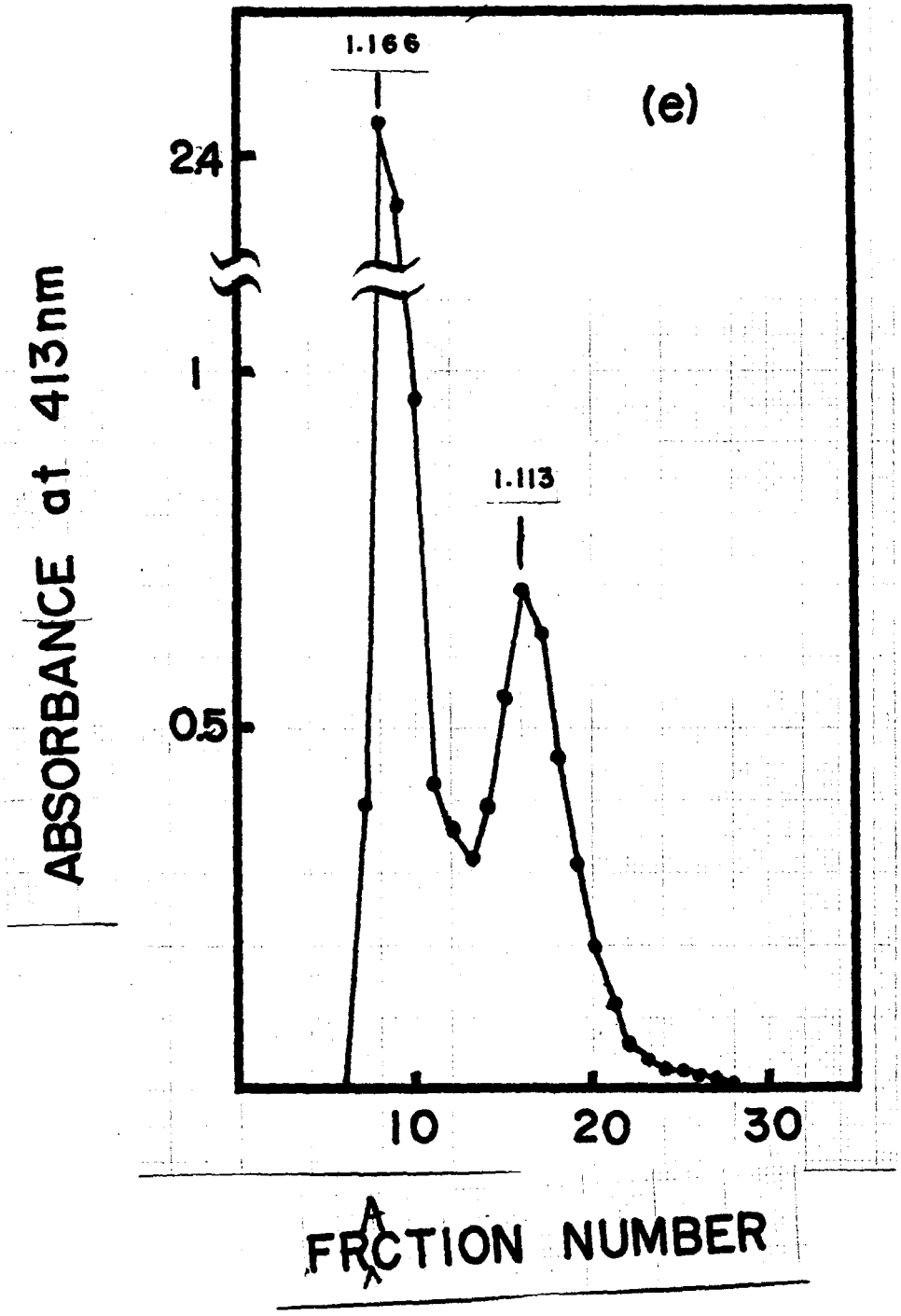

1.143
(C)
1.144
(d)
1.170
ABSORBANCE at 413nm
1
0.5
10
20
30
FRCTION NUMBER

Fig. 1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(e)
2.4
1
0.5
1.113
ABSORBANCE at 413nm
10
20
30
FRCTION NUMBER

Fig. 1

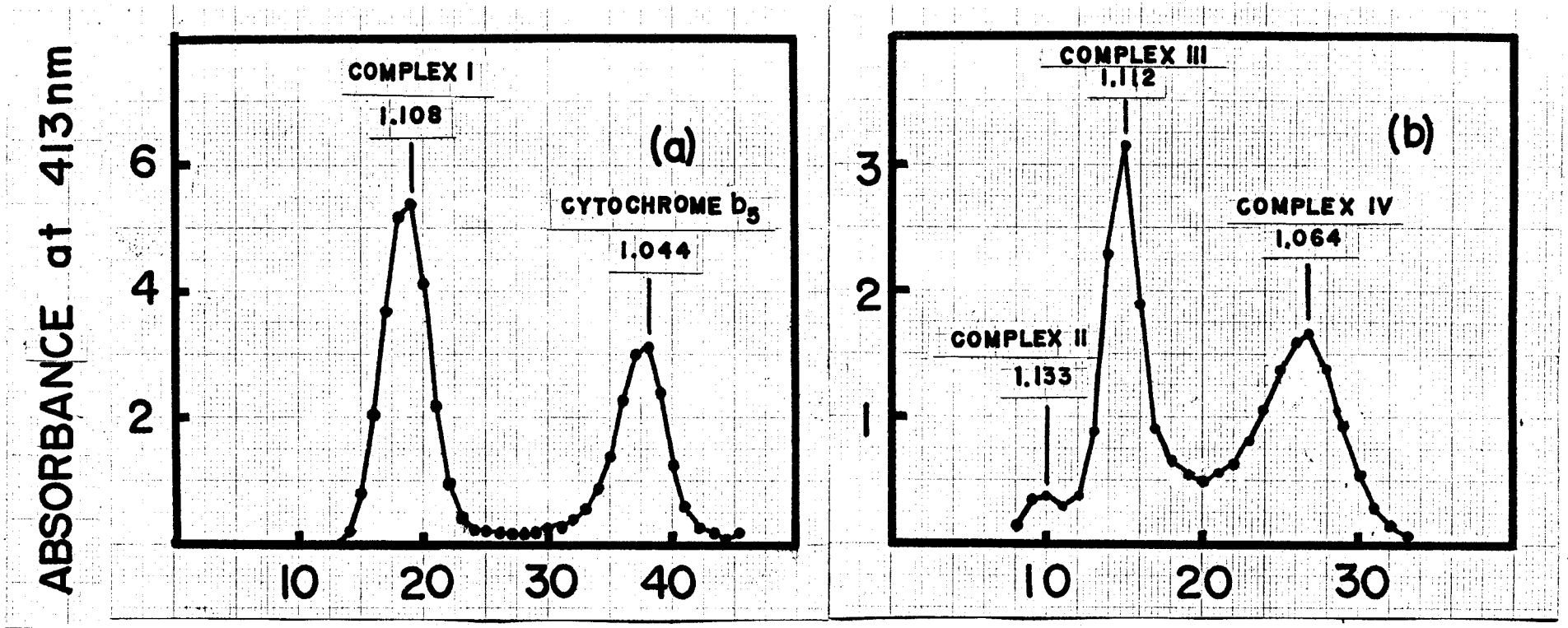
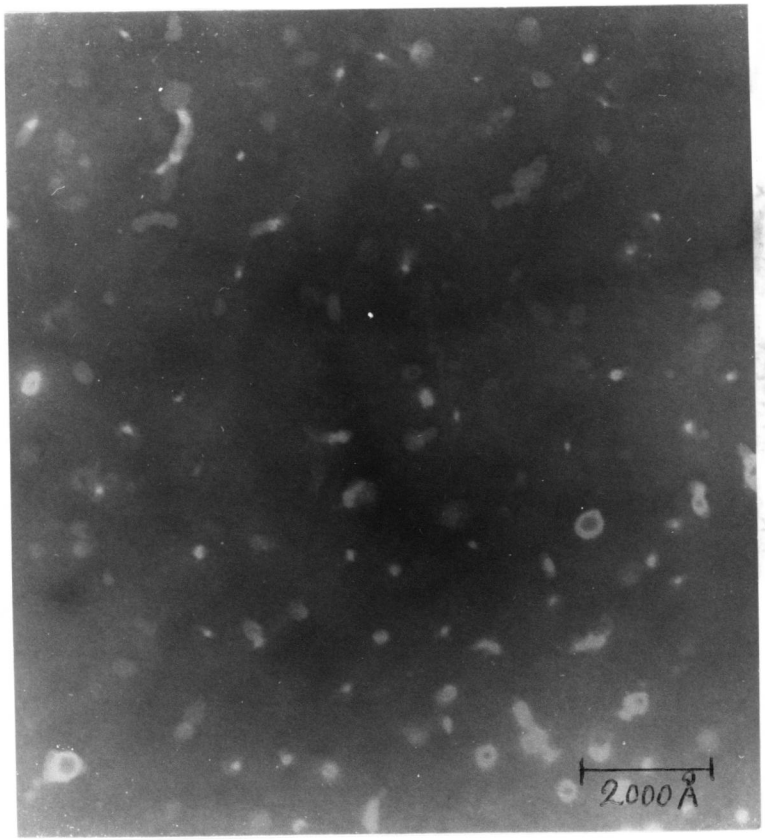

ABSORBANCE at 413nm
COMPLEX I
1.108
(a)
CYTOCHROME b_5
1.044
6
4
2
10
20
30
40
COMPLEX III
1.112
(b)
COMPLEX IV
1.064
COMPLEX II
1.133
3
2
1
10
20
30
FRACTION NUMBER

Fig.2

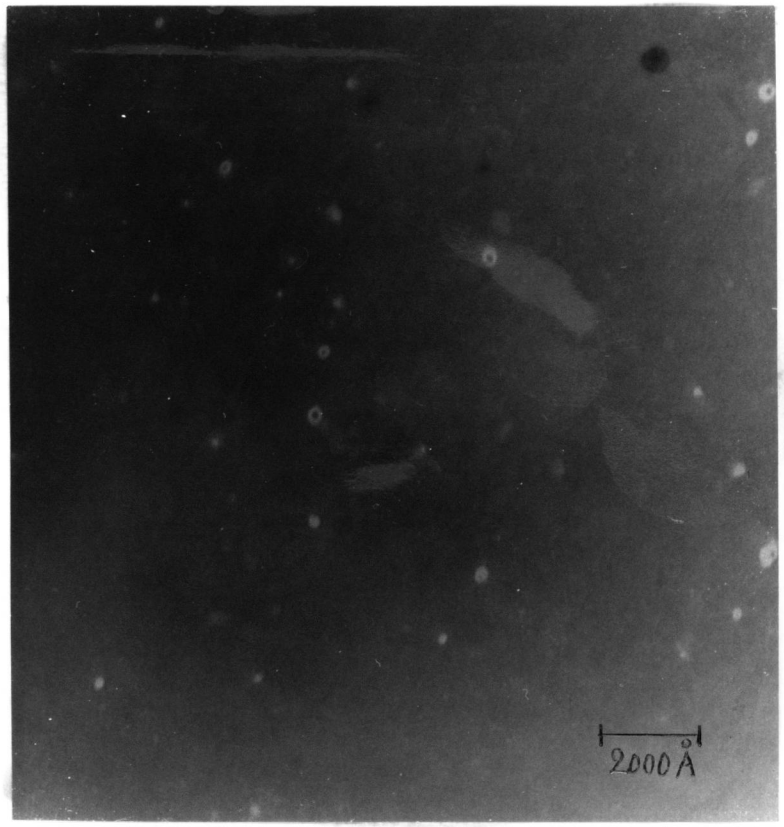
TABLE I. Densities and molar ratios in the four types of complexes

Complex	density	determined molar ratio	calculated molar ratio
I	1.17	10–11	10.8
II	1.15	15	14.4
III	1.12	23	21.6
IV	1.08	50	37.5

Phospholipid and cytochrome $\underline{b}_5$ in the complexes were assayed by measuring phospholipid phosphorus and the absorption of Soret band at 413 nm respectively.

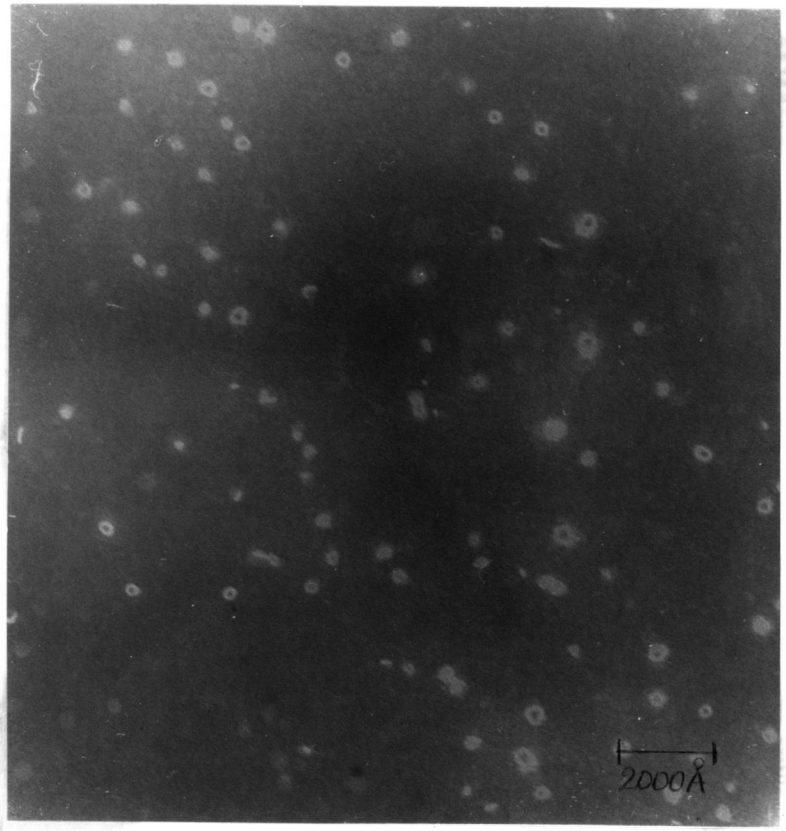


(a)

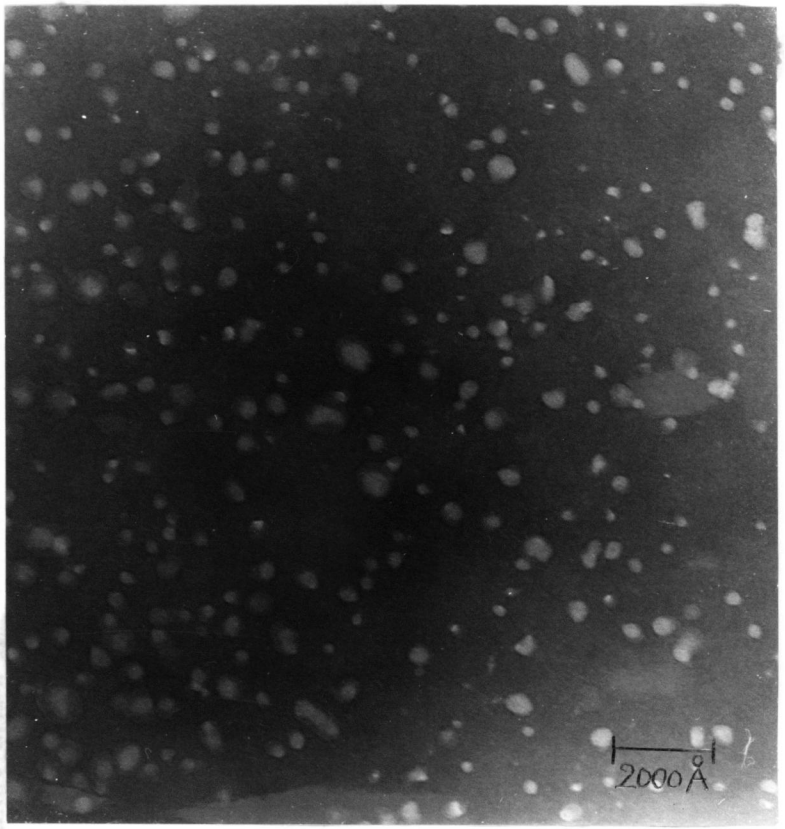


(b)

Fig. 3

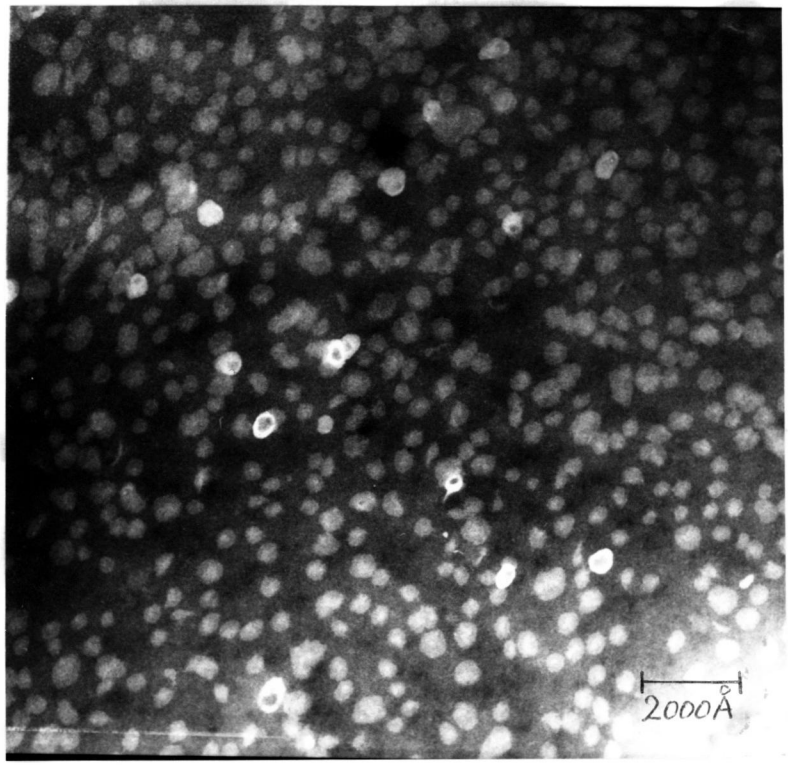


(c)

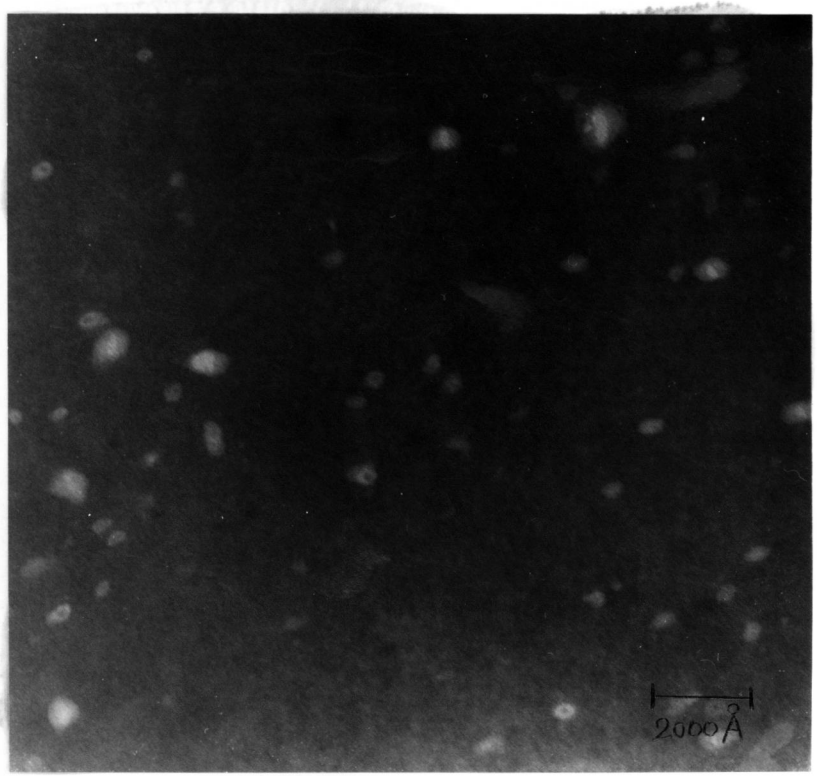


(d)

Fig. 3



(e)



(f)

Fig. 3

TABLE II. Exclusive localization of cytochrome $\underline{b}_5$ on the outer surface of the complexes.

added substances	reduced cytochrome $\underline{b}_5$ (%)
cytochrome $\underline{b}_5$ reductase + NADH	101
$Na_2S_2O_4$ + Triton X-100	100

Cytochrome $\underline{b}_5$ (6.6 nmoles) bound to Complex I in 2 ml of 50 mM Tris-HCl buffer was reduced by adding 10 ul o lysosome-solubilized NADH-cytochrome $\underline{b}_5$ reductase(373 U/ ml) and 20 ul of 30 mM NADH, and the reduced minus oxidized difference spectrum between 409-424 nm was recorded. To effect complete reduction of cytochrome $\underline{b}_5$, 40 ul of 10 % Triton X-100 and small amount of dithionite($Na_2S_2O_4$) were added and the difference spectrum was recorded in the same way.

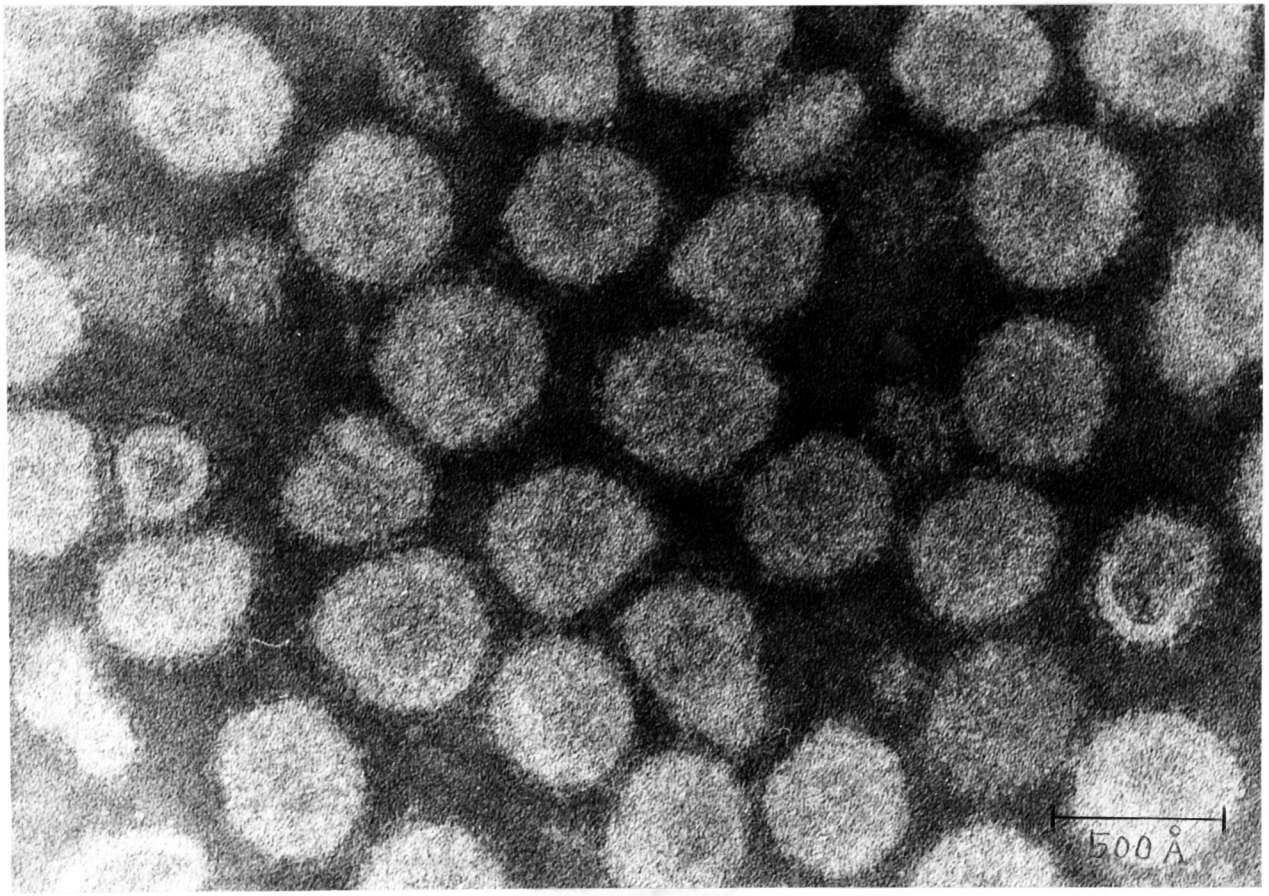


Fig.4

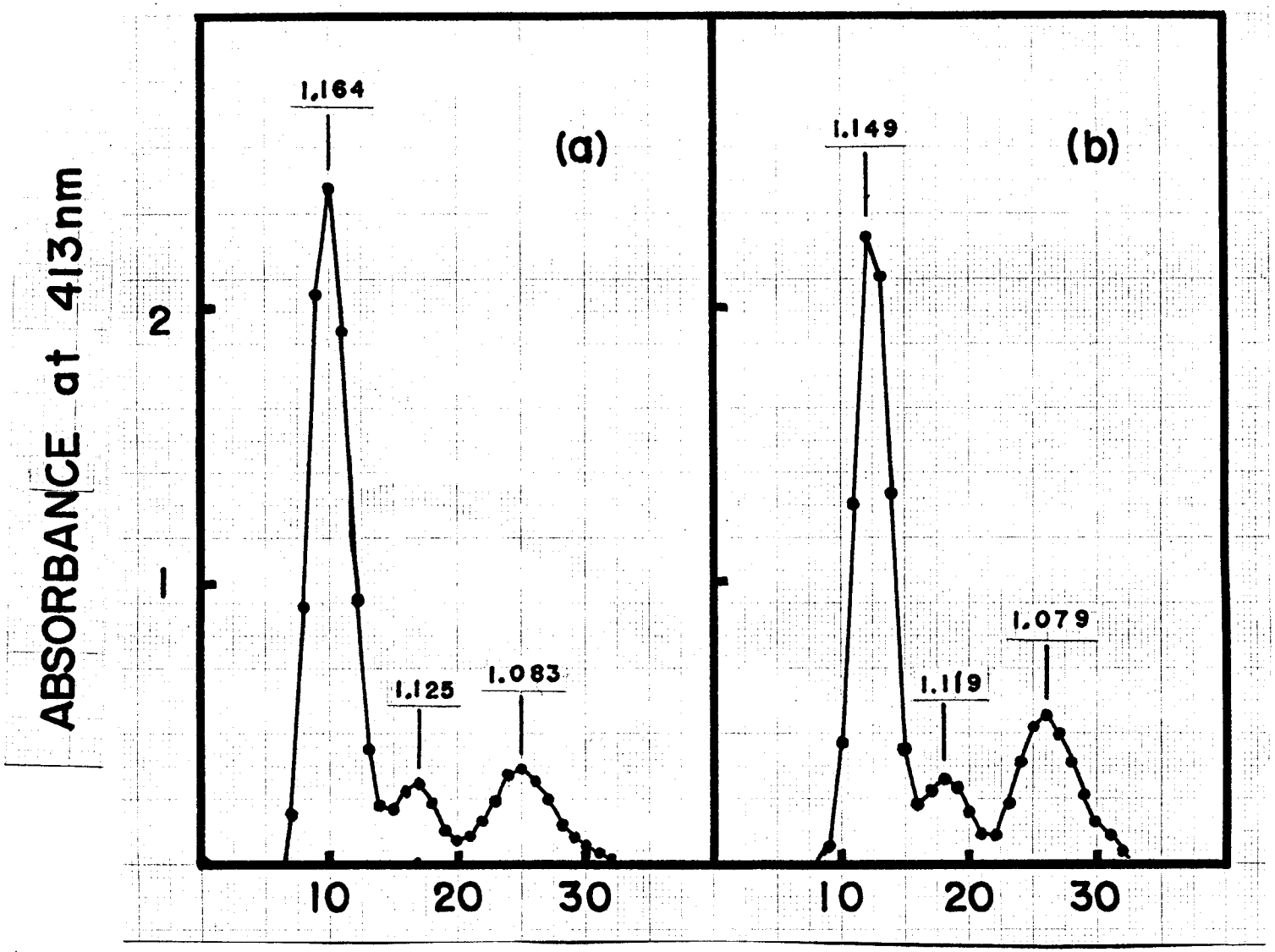

ABSORBANCE at 413nm
2
1
(a)
1.164
1.125
1.083
10
20
30
(b)
1.149
1.119
1.079
10
20
30
FRACTION NUMBER

Fig. 5

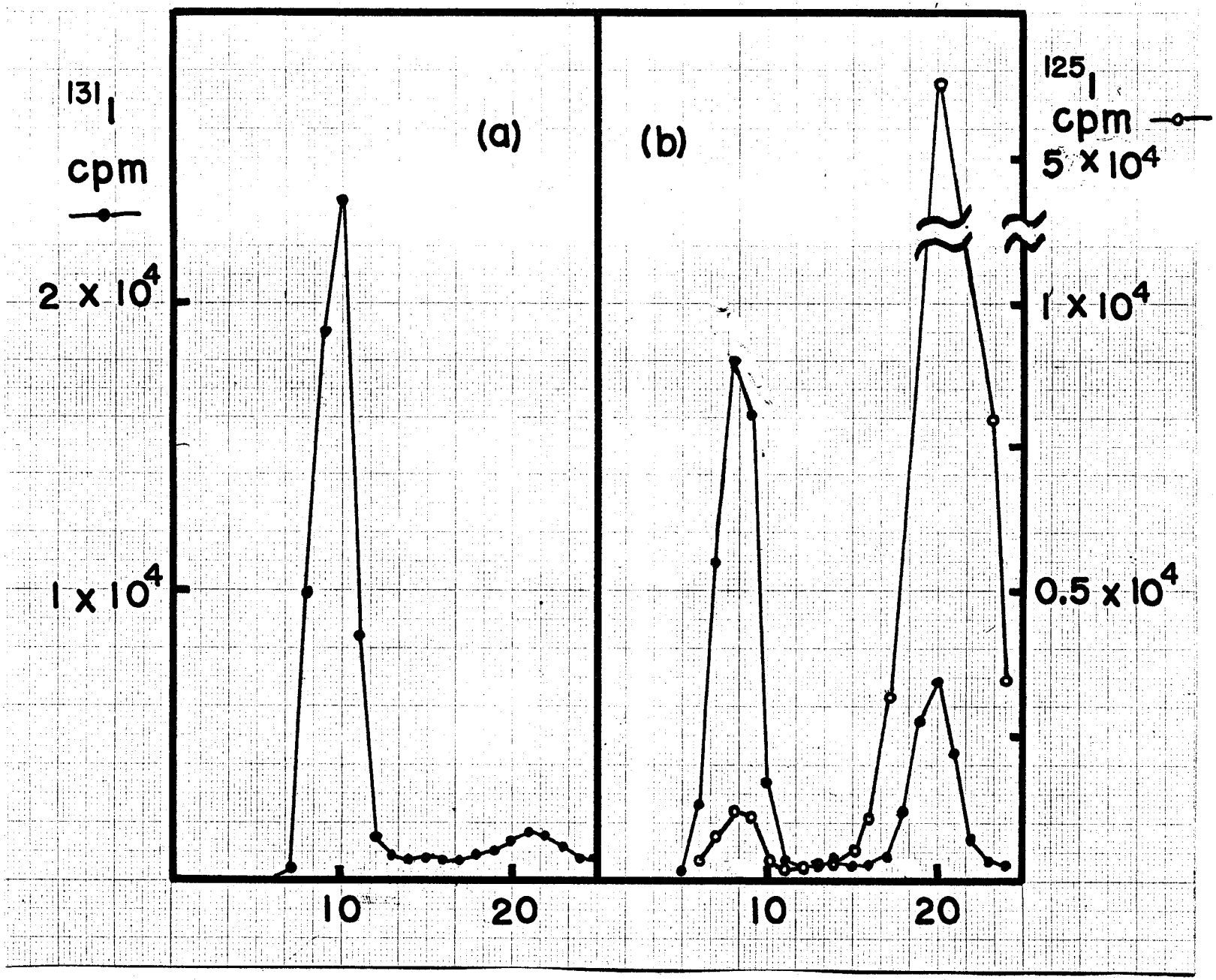

^{131}I
cpm
2 x 10^4
1 x 10^4
(a)
(b)
^{125}I
cpm
5 x 10^4
1 x 10^4
0.5 x 10^4
10
20
10
20
FRACTION NUMBER

Fig.6