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# TRANSFER OF CYTOCHROME $b_5$ AND NADPH CYTOCHROME *c* REDUCTASE BETWEEN MEMBRANES *.

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## Summary

NADPH-cytochrome *c* reductase also reduces cytochrome $b_5$. The reduction is very slow when the proteins are in solution or bound to different membranes. Only when both proteins share a common membrane, is cytochrome $b_5$ reduced rapidly by NADPH. The difference in reaction rates indicates recombination on a common membrane of cytochrome $b_5$ and NADPH reductase originally bound to different vesicles. The recombination of the two proteins occurs with a variety of biological membranes (previously enriched with either reductase or cytochrome $b_5$) as well as with liposomes. We explain this process as protein transfer rather than vesicle fusion for several reasons:

1. The vesicles do not alter shape or size during incubation.
2. The rate of this process corresponds to the rate of incorporation of the single proteins into liposomes carrying the 'complementary' protein.
3. The exchange of proteins between biological membranes and liposomes occupied by protein does not change the density of either membrane.

Protein transfer between membranes appears to be limited to those proteins which had spontaneously recombined with a preformed membrane. In contrast, proteins incorporated into liposomes by means of a detergent were not transferred, nor were endogenous cytochrome $b_5$ and NADPH-cytochrome *c* reductase transferred from microsomes to Golgi membranes or lipid vesicles.

We conclude that the endogenous proteins and proteins incorporated in the

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Abbreviations: TED buffer, 0.1 M Tris-HCl (pH 7.6)/$10^{-3}$ M EDTA/$10^{-3}$ M dithiothreitol; EPL, essential phospholipids.

presence of a detergent are linked to the membrane in another manner than the same proteins which had been inserted into a preformed membrane.

## Introduction

Cytochrome $b_5$ and the NADPH-cytochrome *P*-450 (cytochrome *c*) reductase (EC 1.6.2.4) both belong to the electron transport chains of the endoplasmic reticulum. Although cytochrome $b_5$ is generally reduced by NADH via a specific flavoprotein [1], NADH-cytochrome $b_5$ reductase (EC 1.6.2.2), it also accepts electrons from the NADPH-cytochrome *P*-450 reductase [2] which it then transfers to the fatty acid desaturase (EC 1.14.99.5) or cytochrome *P*-450 [3,4]. This reduction occurs readily in microsomes but is very slow between the solubilized proteins, even at high salt concentrations which increase the reaction rate [5]. Studies with a reconstituted system have shown that the NADPH reductase and cytochrome $b_5$ incorporated into the same membrane interact promptly, whereas the electron transfer between the two proteins is very slow when they belong to separate membranes [6].

Since the rate of cytochrome $b_5$ reduction by the NADPH reductase is so much higher when the two proteins share a common membrane instead of being bound to separate membranes, this system is a convenient tool to investigate membrane-membrane interactions resulting in the appearance of rapidly reducible cytochrome $b_5$. The same principle has been used by Enoch et al. [7] with a system consisting of cytochrome $b_5$, NADH-cytochrome $b_5$ reductase and phospholipid vesicles.

The experiments described below were concerned with the following questions: Is there an interaction between membranes occupied by cytochrome $b_5$ and membranes occupied by NADPH reductase which leads to the reassembly of the two proteins on a common membrane? If such a reassembly can be shown, is it due to vesicle fusion or to protein transfer between membranes? Is such a reaction limited to simple liposomes or is it possible with biological membranes as well? Does it occur with artificially incorporated proteins only or also with endogenous proteins of native membranes?

## Materials and Methods

Cytochrome *c* and dithiothreitol were obtained from Sigma (St. Louis, MO). NADPH was a product of Boehringer (Mannheim). Essential phospholipids (EPL) (mainly phosphatidylcholine with unsaturated fatty acids) were a gift of Professor Dr. J. Fox (Nattermann, Köln). Ultrogels AcA 34 and AcA 22 were manufactured by Industrie Biologique Française. Sephadex G-25 and G-75, and DEAE-Sephadex A-50 were purchased from Pharmacia (Uppsala).

Liver microsomes from phenobarbital-induced rabbits were prepared following the procedure of Frommer et al. [8].

Lipids were extracted from rabbit or pig liver microsomes as described by Holtzman and Gillette [9] and stored in chloroform at −30° C for up to 4 weeks.

NADPH-cytochrome *c* (*P*-450) reductase and cytochrome $b_5$ were isolated

simultaneously from rabbit liver microsomes solubilized with Renex 690 according to Dignam and Strobel [10]. The reductase was partially purified as described by these authors with a minor modification: instead of DEAE-Sephadex A-25, DEAE-Sephadex A-50 was used for ion-exchange chromatography of the microsomal extract, because it retained cytochrome $b_5$. The purification procedure was followed up to the chromatography on agarose A 0.5 m. The resulting reductase preparation reduced about 10 μmol cytochrome *c*/min per mg. It was free of cytochromes *P*-450 and $b_5$ and of NADH-cytochrome $b_5$ reductase. SDS gels showed 2—4 weak, additional bands and it still contained phospholipid. Renex 690 was removed by treatment with Biobeads SM 2 [11].

The cytochrome $b_5$ fractions, eluted from DEAE-Sephadex A-50 with 0.14 M KCl in 0.1 M Tris-HCl/$10^{-3}$ M EDTA/$10^{-4}$ M dithiothreitol (pH 7.6, containing 0.12% Renex 690 and 20% glycerol) were concentrated by ultrafiltration in a GN-10-400 cell by Berghof and further purified by gel filtration through Sephadex G-75 equilibrated with 0.25 M KSCN/0.23% deoxycholate in 0.1 M Tris/acetate (pH 8.1) [12]. This step was repeated twice until the resulting cytochrome $b_5$ was free of phospholipid and gave a single band on disc gel electropherograms. (In several cases, an additional, small band was found in front of the main band. We assume this band to be also cytochrome $b_5$, though modified by proteolytic attack.) Deoxycholate was removed by gel filtration through Sephadex G-25.

Golgi membranes were a gift from Dr. M. Gratzl. They had been prepared according to Ehrenreich et al. [13] as modified by Gratzl and Schwab [14]. The fractions banding at the interface to 0.86 M and 1.12 M sucrose were designated $GF_2$ and $GF_3$, respectively. They were kept at −30° C in 0.01 M cacodylate (pH 7.4) containing the sucrose from the sucrose gradient and were resuspended in 0.1 M Tris-HCl (pH 7.6)/$10^{-3}$ M EDTA/$10^{-3}$ M dithiothreitol (TED buffer) before use.

Inner membranes from *Pseudomonas putida*, prepared according to Ref. 15, were given by Dr. Vera Seydewitz.

Liposomes were prepared by the method of Huang [16]: 60 mg EPL or microsomal lipids were swollen in 3 ml TED buffer and sonicated for 30 min. The pH was maintained by addition of 1 drop 2 N NaOH. Metal particles from the probe were removed by 10 min centrifugation at low speed. The formation of single-walled liposomes was ascertained by freeze-fracture electron microscopy.

Liposomes filled with ferricyanide were obtained by sonicating the lipids in 1 M $K_3Fe(CN)_6$, dissolved in TED buffer. External solute was removed by gel chromatography on Sephadex G-25 in the same buffer.

Cytochrome $b_5$ and the reductase were bound to biological membranes or liposomes by incubation for 1 h at 30° C in TED buffer. When inserted into biological membranes, the bound proteins were separated from unbound proteins by 1 h centrifugation at 100 000 × *g*. The sedimented membranes were washed once and resuspended in TED buffer. When the proteins were bound to liposomes, gel filtration through Ultrogels AcA 34 (cytochrome $b_5$) and AcA 22 (reductase) separated the bound from the unbound proteins [17]. Only the first half of the lipoprotein peak was used for the recombination experi-

ments described below in order to avoid contamination by traces of unbound protein. On the average, cytochrome $b_5$ was bound to lipid in a molar ratio of 1 : 200 and NADPH reductase was bound to lipid in a molar ratio of 1 : 3000.

In some instances the proteins were reconstituted with lipids in the presence of cholate as described by Brunner et al. [18] for the formation of liposomes: 20 mg/ml lipid, swollen in TED buffer, was clarified by addition of 0.1 vol. 20% sodium cholate and mixed with an appropriate amount of protein (containing 1% cholate). The mixture was applied to a column (1.5 × 30 cm) of Ultrogel AcA 34 (cytochrome $b_5$) or Ultrogel AcA 22 (reductase) and eluted with TED buffer. This step separated the resulting lipoproteins from the detergent as well as from unbound protein. Residual cholate was removed by dialysis against TED buffer.

The recombination on a common membrane of cytochrome $b_5$ and NADPH reductase bound previously to separate membranes was achieved by incubating the mixture at 30° C.

$GF_2$ and microsomes were rapidly separated by density gradient centrifugation in the airfuge (Beckman): The incubation mixture was made 25% with respect to sucrose. 0.15 ml were pipetted into each tube and overlayered by 0.01 ml TED buffer. 10 min centrifugation at 160 000 × $g$ sedimented the microsomes and yielded the Golgi fraction as a sharp whitish band in the upper third of the gradient, which was collected by means of a syringe.

*Analytical procedures.* SDS gel electrophoresis was carried out as described by Glossmann and Lutz [19].

Phospholipid was determined as inorganic phosphate after digestion with sulfuric acid by the method of Bartlett [20]. Protein was estimated according to Lowry et al. [21], using bovine serum albumin as standard. The assay of NADPH-cytochrome *c* reductase followed the procedure of Phillips and Langdon [22], but with 0.33 M phosphate buffer instead of 0.05 M buffer. An extinction coefficient of 21 000 $M^{-1} \cdot cm^{-1}$ was used for the increase of absorbance at 550 nm.

Cytochrome $b_5$ was assayed as described by Takesue and Omura [23]. In pure samples it was determined by its absorbance at 413 nm, using an extinction coefficient of 117 000 $M^{-1} \cdot cm^{-1}$ [24]. The rapid enzymatic reduction of cytochrome $b_5$ was measured by the increase of the absorbance at 423 nm following the addition of NADPH (final concentration 0.2 mM) to a sample suspended in 0.2 M potassium phosphate buffer (pH 7.4). Total reduction of cytochrome $b_5$ was achieved by addition of solid sodium dithionite. 100 000 $M^{-1} \cdot cm^{-1}$ was taken as extinction coefficient [1].

## Results

When liposome-bound reductase was incubated with liposome-bound cytochrome $b_5$, part of the cytochrome could be rapidly reduced by NADPH, apparently in the same manner as when the two proteins were bound to the same vesicles. (The reduction was too fast for the kinetics to be determined by conventional methods; these will be dealt with in a separate paper.) The percentage of rapidly reducible cytochrome $b_5$ varied between 20 and 60% depending on the ratio of reductase to cytochrome. The same is the case, when

both proteins are incorporated simultaneously into the same membrane (Fig. 1): the rapid enzymatic reduction of cytochrome $b_5$ never reached the level attained in microsomes, with one exception: when cytochrome $b_5$ was reconstituted with vesicles carrying reductase, almost 90% were reducible by NADPH (Fig. 3b).

This increase in rapidly reducible cytochrome indicated a reunion of the two proteins on the same membrane, which could be explained either by vesicle fusion or by a protein transfer between different vesicles. We therefore looked for an increase in vesicle size in the incubation mixture. But freeze-fracture electron micrographs (not shown) revealed no visible change in vesicle appearance after 5 h incubation at 0° C which resulted in 25% of the cytochrome being rapidly reducible by NADPH. Although this experiment did not completely rule out vesicle fusion (if only two vesicles fuse, the diameter of the resulting vesicle is only $\sqrt[3]{2}$ times that of the original liposomes), it rendered this mechanism unlikely. Thus, like Enoch et al. [7] concluding from their gel filtration study, we presumed that the proteins can be transferred from one membrane to another membrane.

*Time dependence of the protein transfer*

To confirm this conclusion further, we compared the rate of this process to the rate of incorporation of either cytochrome $b_5$ or the reductase into liposomes carrying reductase or cytochrome $b_5$, respectively. Both processes were monitored by the increase of rapidly reducible cytochrome $b_5$. The protein exchange between liposomes occurred even at low temperatures. At 0° C, half-maximum effect was obtained after 5 h, at 15° C it was reached after 2 h, and at 30° C after 15 min (Fig. 2). The NADPH-cytochrome *c* reductase activity did not decline during long periods of incubation at 30° C. To the contrary: in most experiments it increased to 130—140% of the original activity after 5 h

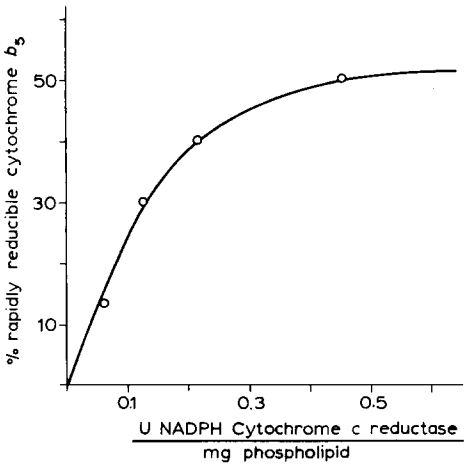


**Fig. 1. Correlation of liposomal reductase concentration with percentage of rapidly reducible cytochrome $b_5$. Samples with increasing amounts of reductase and 8 nmol cytochrome $b_5$/mg phospholipid were assayed for rapidly reducible cytochrome $b_5$. The proteins had been incorporated simultaneously into the same liposomes from microsomal lipids.**

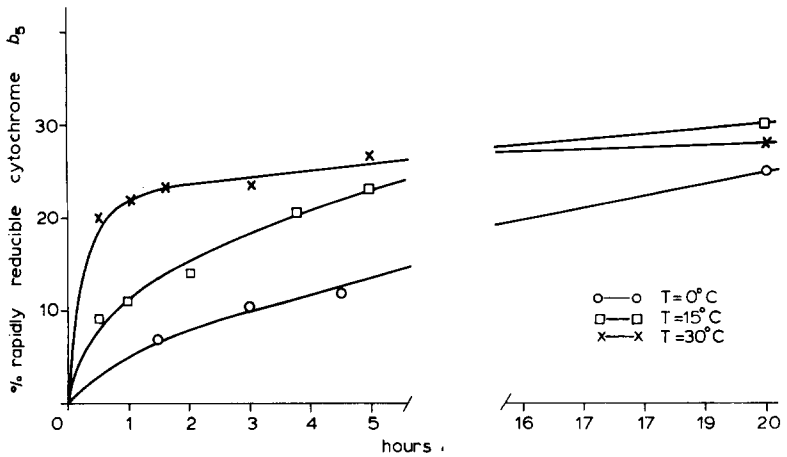


Fig. 2. Time course of protein transfer at different temperatures. 0.135 U reductase and 4.7 nmol cytochrome $b_5$, bound separately to liposomes from microsomal lipids, were incubated at the temperatures indicated above. Beginning with $t = 0$, aliquots were taken at suitable time intervals and assayed for rapidly reducible cytochrome $b_5$.

at 30°C. At 30°C, the rate of protein exchange apparently did not depend on vesicle size or the nature of the membrane used: it was practically always the same, regardless, whether the protein had been bound to liposomes consisting of microsomal lipids or EPL or whether they had been bound to biological membranes such as Golgi membranes or the inner membranes of *P. putida* (Fig. 3a). The half-time of the exchange corresponded roughly to the half-time of incorporation of cytochrome $b_5$ into liposomes carrying NADPH-cytochrome *c* reductase or, vice versa, of reductase into liposomes occupied by cytochrome $b_5$ (Fig. 3b).

*Exchange of cytochrome $b_5$ and reductase bound to vesicles of different density*

In order to demonstrate exchange of cytochrome $b_5$ as well as of NADPH reductase it was imperative to separate the different vesicle populations after incubation of the mixture. To this purpose, cytochrome $b_5$ was incorporated into liposomes and the reductase was bound to Golgi membranes which could be easily separated from liposomes by density gradient fractionation.

During incubation of the mixed vesicle populations part of the cytochrome became rapidly reducible by NADPH. Fractionation of the incubation mixture revealed that about 30% of the reductase originally bound to Golgi membranes were to be found in the same fractions as the liposome-bound cytochrome, whereas 20% of the cytochrome $b_5$ reappeared in the Golgi fraction (Fig. 4). All the 'high-density' cytochrome was quickly reduced by NADPH. It could be excluded that the protein reassembly on the Golgi membrane was the result of fusion between $GF_3$ and liposomes: The ratio phospholipid : protein is not altered by incubation of $GF_3$ with cytochrome $b_5$-liposomes, whereas it increases considerably on incubation of $GF_3$ with protein-free liposomes.

When Golgi membranes carrying cytochrome $b_5$ were incubated with lipo-

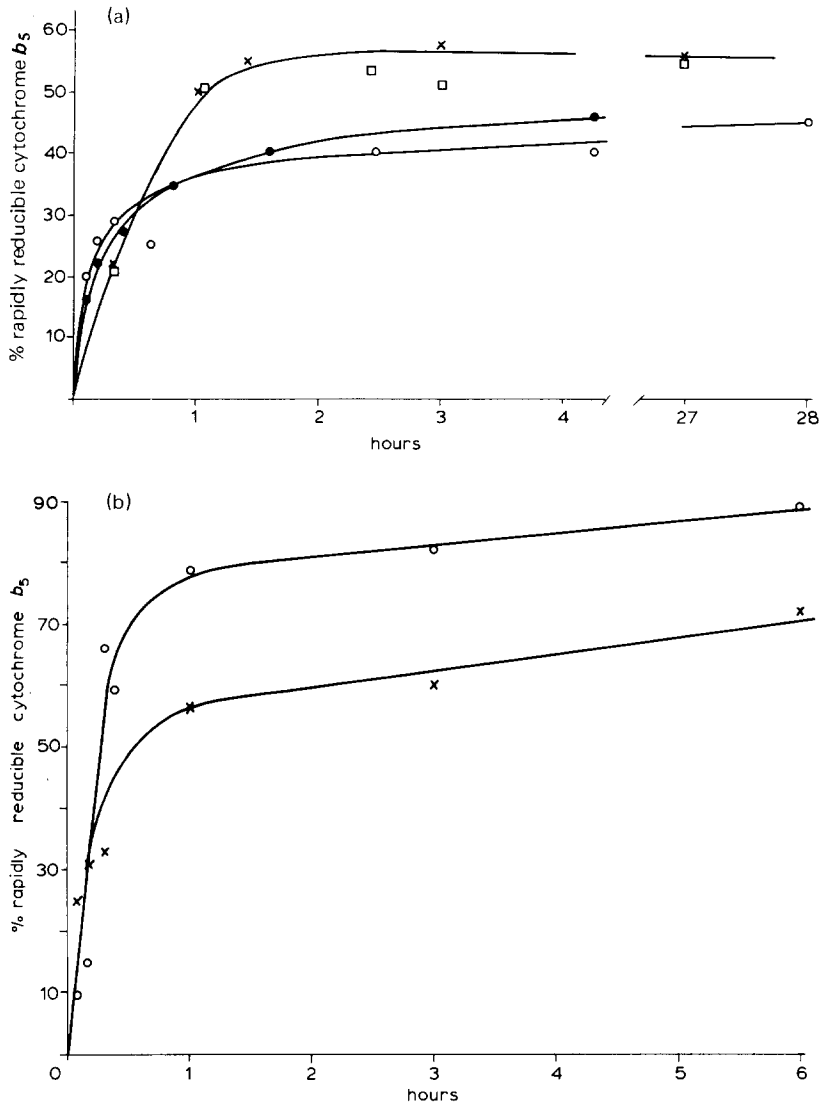


Fig. 3. Time course of the increase in rapidly reducible cytochrome $b_5$. (a) 1 ml of the following mixtures were incubated at 30°C: X———X, 0.45 U liposome-bound reductase + 4.5 nmol $GF_3$ cytochrome $b_5$; □———□, 0.2 U $GF_3$ reductase + 8 nmol $GF_3$ cytochrome $b_5$; ●———●, 0.09 U liposome-bound reductase + 3.1 nmol liposome-bound reductase; ○———○, 0.45 U reductase, bound to inner membranes from *P. putida*, + 4.5 nmol cytochrome $b_5$, bound to EPL liposomes. At $t = 0$ and suitable time intervals, aliquots were assayed for rapidly reducible cytochrome $b_5$. (b) ○———○, 7.3 nmol/ml cytochrome $b_5$ was incubated with 0.69 U/ml reductase bound to EPL liposomes; X———X, 0.47 U/ml reductase was incubated with 4.7 nmol/ml cytochrome $b_5$ bound to EPL liposomes. At the times indicated, aliquots were assayed for rapidly reducible cytochrome $b_5$.

somes occupied by the reductase, 33% of the cytochrome reappeared in the liposome fraction and about 10% of the reductase was found in the Golgi fraction (Fig. 5). This part of the experiment was more difficult to evaluate, since part of the liposome-bound reductase overlapped with the Golgi fraction. The amount of transferred protein in a given fraction is calculated as the

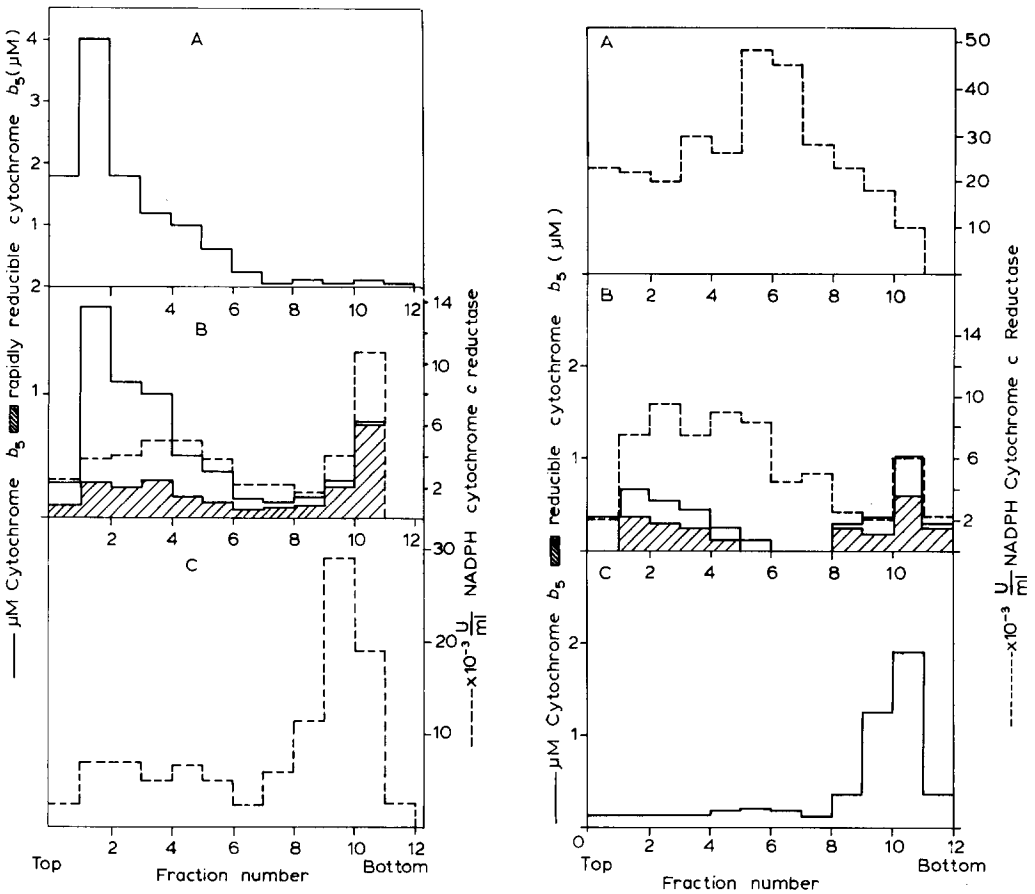


Fig. 4. Transfer of reductase and cytochrome $b_5$ between Golgi membranes and liposomes. 0.12 U reductase was bound to Golgi membranes (2.4 mg protein) previously treated with neuraminidase from *Vibrio cholerae*. The membrane-bound reductase was incubated for 3.5 h at 30°C with 8 nmol cytochrome $b_5$ bound to liposomes from microsomal lipids. The mixture was fractionated in a 5—35% sucrose gradient. 1 ml fractions were collected. (A) Distribution of 8 nmol liposome-bound cytochrome $b_5$. (B) Distribution of cytochrome $b_5$ and reductase of the incubation mixture. (C) Distribution of 0.12 U $GF_3$ reductase.

Fig. 5. Transfer of cytochrome $b_5$ and reductase between Golgi membranes and liposomes. 4.2 nmol cytochrome $b_5$ was bound to Golgi membranes (2.4 mg protein) previously treated with neuraminidase from *V. cholerae*. The bound cytochrome was incubated for 3.5 h at 30°C with 0.43 U reductase bound to liposomes from microsomal lipids. The mixture (containing 4.2 nmol cytochrome $b_5$ and 0.15 U reductase) was centrifuged in a 5—35% sucrose gradient. 1 ml fractions were collected. (A) Distribution of 0.28 U liposome-bound reductase. (B) Distribution of reductase and cytochrome $b_5$ of the incubation mixture. (C) Distribution of 4.2 nmol $GF_3$ cytochrome $b_5$.

amount in excess of that to be found in the corresponding fraction of a control sample, in this case cytochrome $b_5$-$GF_3$ and reductase-liposomes centrifuged separately (corrected for yield).

So far these experiments were performed with proteins inserted into preformed membranes. The question arose whether proteins incorporated otherwise could also be exchanged between membranes. There are reconstitution

methods which integrate protein and lipid in the presence of detergents such as cholate [25]. In such methods, the protein-lipid membrane is formed on removal of the detergent. Preliminary experiments with $^3$H-labeled sodium cholate have shown that the detergent is effectively removed from these preparations by gel filtration and subsequent dialysis: The residual detergent was one cholate per 3000 phospholipid molecules.

The structure of the cholate-prepared EPL membranes appears to be heterogeneous. Freeze-fracture electron micrographs revealed tiny, closed vesicles along with a few large, disordered fragments. Gel filtration through Sepharose 4B demonstrated that such lipoproteins (made with cytochrome $b_5$) enclose potassium ferricyanide, if prepared in the presence of ferricyanide. But in these samples the molar ratio ferricyanide : phospholipid was only 0.02, whereas in comparable cytochrome $b_5$ liposomes (sonicated) it was 0.07, although the latter were leaky. About 80% of the heme-containing part of cytochrome $b_5$ could be split off by trypsin and more than 90% of the cytochrome was reduced by NADH via a crude lysosome-prepared NADH-cytochrome $b_5$ reductase, thus establishing that practically all the protein was oriented to the outside of the vesicles just as in lipoproteins prepared with sonicated liposomes. A similar reasoning applies to the NADPH reductase: only the molecules oriented to the outside react with cytochrome *c*. Since there was no activity missing after recombination by the cholate method, all the reductase must have access to the cytochrome *c* in the external solution.

When cholate-prepared cytochrome $b_5$ liposomes were incubated with cholate-prepared NADPH reductase liposomes, the cytochrome was not rapidly reduced by NADPH, provided the bound proteins were carefully separated from traces of unbound protein by gel chromatography. In control samples containing both proteins on a common membrane most of the cytochrome was quickly reduced by NADPH. Fig. 6 shows that in a mixture incubated for 90 min only 5% of the total cytochrome $b_5$ was reduced by NADPH indicating that both proteins were still bound to separate vesicles. Accordingly, when microsomes were incubated with cholate-prepared EPL cytochrome $b_5$ (molar ratio 480 : 1) they did not noticeably increase their content of cytochrome $b_5$. As can be seen in Table I, the incubated microsomes had a higher percentage of cytochrome $b_5$ in the low-density fractions than had the control. This indicates an uptake of lipid along with some additional cytochrome $b_5$. But for the major part of the microsomes the cytochrome $b_5$ content of the incubated and the control sample agreed within 10%, whereas in microsomes incubated with comparable, but sonicated cytochrome $b_5$ liposomes the cytochrome $b_5$ content of all fractions had markedly increased.

The next experiments were performed with freshly prepared microsomes and (aged) Golgi membranes in order to see whether a transfer of endogenous NADPH reductase could be observed from the microsomes to the Golgi fraction. Fresh microsomes were incubated for 2 h at 30° C with Golgi membranes ($GF_2$) and then quickly separated from the Golgi membranes by high-speed centrifugation in the Beckman airfuge. This yielded the Golgi fraction as a sharp whitish band in the upper third of the tube, whereas the microsomes were sedimented. An intermediate fraction, mainly derived from the microsomes, was discarded. Subsequently, the Golgi membranes and microsomes

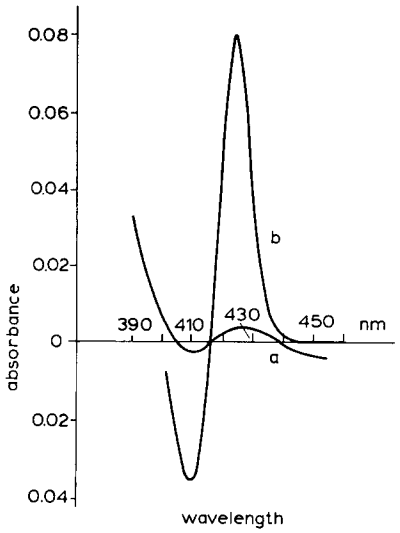


Fig. 6. Reducibility of cytochrome $b_5$ following the incubation of cholate-prepared EPL cytochrome $b_5$ with cholate-prepared EPL reductase. 0.114 U EPL reductase (cholate prepared) was incubated for 90 min at 30°C with 2.5 nmol EPL cytochrome $b_5$ (cholate prepared). The figure shows the difference spectrum of reduced minus oxidized cytochrome $b_5$ following the addition of (a) NADPH and (b) $Na_2S_2O_4$.

were refractionated in a continuous sucrose gradient and analysed for NADPH-cytochrome *c* reductase: The thoroughly purified Golgi membranes exhibited only traces of reductase activity due to their own residual reductase. No NADPH-cytochrome *c* reductase had been transferred from microsomes to the Golgi membranes (data not shown). Neither was it extracted from microsomes by EPL liposomes carrying cytochrome $b_5$ (Fig. 7A). Only when additional reductase had previously been bound to the microsomes, part of the activity was transferred to cytochrome $b_5$ liposomes (Fig. 7B). Accordingly, only artificially bound cytochrome $b_5$ was transferred from microsomes to protein-

TABLE I

CYTOCHROME $b_5$ CONTENT OF MICROSOMES INCUBATED WITH CHOLATE-PREPARED CYTOCHROME $b_5$ LIPOSOMES

Microsomes (2.4 mg protein, 1.5 nmol cytochrome $b_5$/mg)(this unusually high value is probably due to extensive washing of the microsomes, during which some protein may have been lost.) were incubated for 90 min at 30°C with 4.1 nmol cytochrome $b_5$ bound to EPL liposomes with the cholate method. The incubation mixture was fractionated in a sucrose gradient. An identical mixture was fractionated immediately after mixing as control.

| Density (g/ml) | nmol cytochrome $b_5$/mg protein | | % of microsomal cytochrome $b_5$ * | |
|---|---|---|---|---|
| | Incubated | Control | Incubated | Control |
| 1.14 | 2 | 1.8 | 25.1 | 11.9 |
| 1.15 | 1.6 | 1.8 | 60.2 | 47.3 |
| 1.20 | 1.3 | 1.0 | 5.3 | 28.9 |

* Only the three main fractions were considered, therefore the numbers do not add up to 100%.

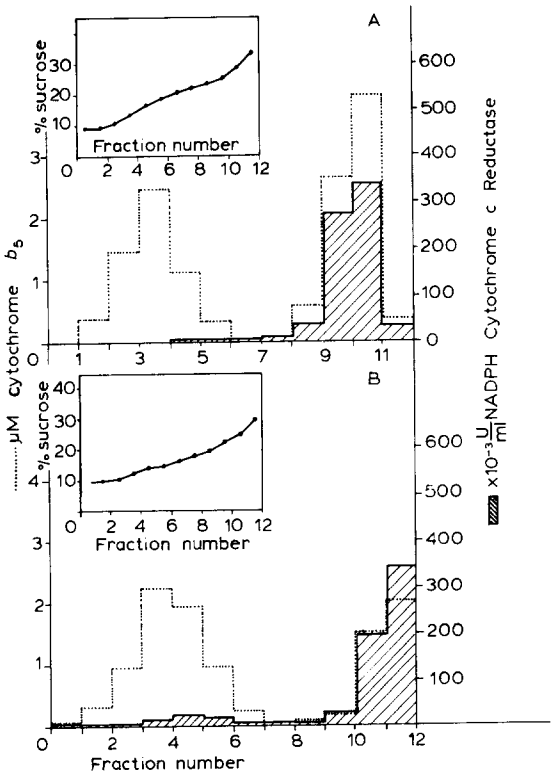


Fig. 7. Transfer of artificially bound reductase from microsomes to liposomes carrying cytochrome $b_5$. (A) Microsomes (3.8 mg protein, 0.2 U reductase/mg) were incubated for 90 min at 30°C with 12.1 nmol EPL cytochrome $b_5$. The mixture was separated in a 5—35% sucrose gradient. 1 ml fractions were collected. Inset: Sucrose concentration. (B) Reductase-enriched microsomes (1.7 mg protein, 0.33 U reductase/mg) were incubated for 90 min at 30°C with 12.1 nmol EPL cytochrome $b_5$ and fractionated as in (A). Inset: Sucrose concentration.

free liposomes, whereas no endogenous cytochrome $b_5$ could be found in liposomes incubated with fresh microsomes (Fig. 8). The distribution of NADPH-cytochrome *c* reductase (also shown in Fig. 8) proves that the appearance of cytochrome $b_5$ in the light fractions was not due to contamination by microsomes.

On incubation with protein-free liposomes the microsomes were shifted to lower densities indicating an uptake of lipid. Extraction and analysis of the microsomal lipids showed that the microsomes had indeed taken up a considerable amount of liposomal lipid. In one experiment, the ratio of phospholipid to protein increased from 0.7 in control microsomes to 1.14 in microsomes incubated with liposomes. No experiment was undertaken to find out how the lipid was attached to the microsomes. It cannot be simply by adsorption, since the control mixture of microsomes and liposomes was easily separated by density gradient centrifugation. It may be similar to the uptake of liposomal lipid by Chinese hamster V79 cells described by Pagano and Huang [26], who suggested fusion as a mechanism.

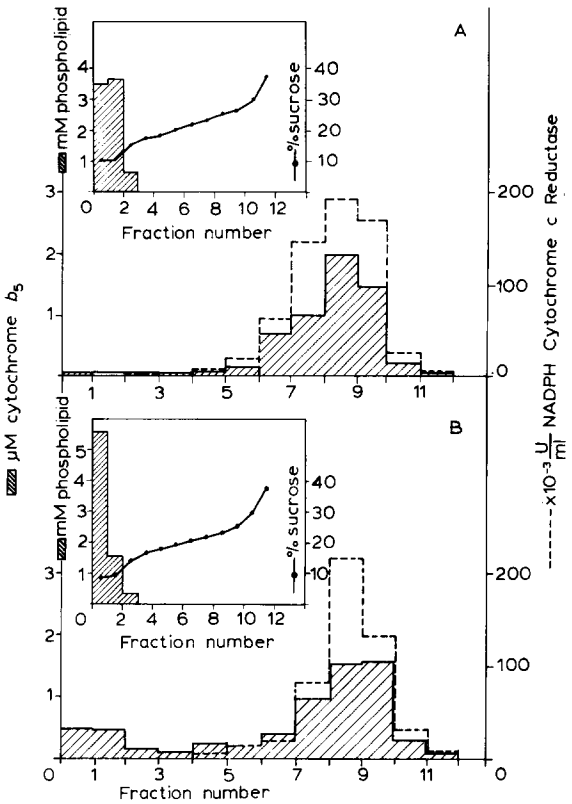


Fig. 8. Transfer of artificially bound cytochrome $b_5$ from microsomes to EPL liposomes. (A) Microsomes (5.1 mg protein, 1.5 nmol cytochrome $b_5$/mg) were incubated for 90 min at 30°C with EPL liposomes (10 µmol EPL), then fractionated in a sucrose gradient. Inset: Distribution of liposomes and sucrose concentration. (B) Cytochrome $b_5$-enriched microsomes (2.5 mg protein, 3.2 nmol cytochrome $b_5$/mg) were incubated for 90 min at 30°C with EPL liposomes (10 µmol EPL) and fractionated as in (A). The inset shows the distribution of EPL and the concentration of sucrose.

On the other hand, microsomes incubated with cytochrome $b_5$-liposomes retained their original density as did Golgi membranes.

## Discussion

As already pointed out by Enoch et al. [7], the increase in the reduction rate of cytochrome $b_5$ inserted into the same membrane as the reductase is probably not only due to facilitated diffusion in the plane of the membrane. It may also result from the favorable orientation of the two proteins with respect to each other. This increase in reduction rate has been used to demonstrate that cytochrome $b_5$ and the NADPH-cytochrome *c* reductase, though initially bound to separate membranes can reassemble on a common membrane. We think that this phenomenon is not the result of vesicle fusion, since freeze-fracture electron micrographs of a mixture of vesicle-bound cytochrome $b_5$ and vesicle-bound reductase did not show a change in shape or size of the vesicles after incubation, although part of the cytochrome $b_5$ became rapidly reducible by

NADPH. Enoch et al. [7], judging from gel filtration studies, arrived at the same conclusion. (Some uptake of lipid by biological membranes must have occurred as well, since a small part of these membranes had a slightly lower density than the controls. But this is easily explained. The protein-carrying liposomes had been carefully separated from unbound protein, but not from protein-free liposomes. The latter are taken up by biological membranes.)

The alternative explanation for protein reassembly on a common membrane is protein transfer between membranes. Transfer of cytochrome $b_5$ between phospholipid vesicles has been shown to occur by Roseman et al. [27]. These authors demonstrated that on incubation of liposome-bound cytochrome $b_5$ with protein-free liposomes a new species appeared with sedimentation characteristics intermediate between those of the free liposomes and the original cytochrome-carrying liposomes. They also proved the absence of fusion by using vesicles filled with ferricyanide and ascorbate, respectively: although the cytochrome was redistributed between the liposomes, the ascorbate did not reduce ferricyanide.

Protein transfer was also indicated by the similarity of the rate of this process and the rate of protein incorporation into liposomes already carrying the 'complementary' protein (both reactions were monitored by the reducibility of cytochrome $b_5$). This also suggests that the protein is transferred through the aqueous phase rather than by collisions between vesicles. It looks as if proteins artificially inserted into preformed membranes are bound reversibly. Thus there must be an equilibrium between bound and unbound proteins in the same manner as there is an equilibrium between the monomeric and oligomeric forms of detergent-free cytochrome $b_5$ [28].

On rechromatography of the reconstituted and fractionated samples on suitable gels no free protein could be detected and the composition of the lipoproteins was practically the same as that of the original samples. Therefore the lipoproteins were assumed to be stable. Conceivably, the concentration of free protein is too low to be detected. The use of biological membranes allowed to separate the membranes from the liposomes and to demonstrate that, indeed, part of the cytochrome or reductase is transferred from liposomes to biological membranes such as Golgi membranes, microsomes or the inner membranes of *P. putida* and vice versa. The experiments in which protein originally bound to biological membranes reappeared in the liposomal fraction (see Fig. 8B) prove that these proteins must have come by transfer into the light fractions. Fusion between liposomes and microsomes or Golgi membranes would have resulted in a membrane of intermediate density, whereas the liposomes carrying the transferred proteins exhibited the same density as before the incubation. Only when protein-free liposomes were incubated with microsomes or Golgi membranes these membranes took up additional lipid, perhaps by fusion. Apparently, the protein bound to liposomes prevents the interaction of liposomes with biological membranes. We think that sterical hindrance is the reason.

Generally, not all cytochrome $b_5$ was reduced rapidly by NADPH reductase, even when both proteins were bound to the same vesicles. One reason presumably was too low a molar ratio of reductase to cytochrome $b_5$, so that there were some vesicles carrying cytochrome $b_5$, but no reductase. Another reason

might be inhomogeneous distribution of the proteins, which could also result in vesicles having only cytochrome $b_5$. The molar ratio of protein to lipid was generally about 1 : 200 for lipocytochrome $b_5$ and 1 : 3000 for liporeductase. Liposomes of about 250 Å diameter consist of 5000—6000 phospholipid molecules and therefore carried on the average 30 cytochrome and two reductase molecules per vesicle. It is also possible that (for unknown reasons) not all cytochrome $b_5$ was available for interaction with the reductase, although both proteins were bound to the same membrane. There is no doubt that the proteins are bound to the membranes from the outside with the catalytic regions protruding from the membrane into the surrounding solution: More than 90% of the membrane-bound cytochrome can be cleaved by trypsin [17], and the reductase can only be assayed when its catalytic part is accessible to the external cytochrome *c*. Reductase buried in the interior of the vesicles would also appear inactive.

No experiment was undertaken to study the role of the lipid in protein transfer. For liposomes we used either microsomal lipids or 'essential phospholipids' which consist mainly of unsaturated phosphatidylcholine. The membranes used contained either phosphatidylcholine (microsomes, Golgi) or phosphatidylethanolamine (*P. putida*) as the main lipid [29—31]. Cytochrome $b_5$ or the reductase could easily be inserted into all these membranes either by direct incorporation of the free proteins or by transfer from liposomes.

Protein transfer could only be demonstrated with protein previously inserted into preformed membranes. When cytochrome $b_5$ or the reductase were reconstituted with lipid by the cholate method, both proteins remained firmly bound to their respective membranes. This is one more argument in favor of protein transfer as opposed to vesicle fusion. These lipoproteins form vesicles, too, and if there was fusion between sonicated liposomes, these cholate-prepared vesicles should also fuse, resulting in rapidly reducible cytochrome $b_5$.

Obviously, lipoproteins prepared in the presence of cholate differ structurally from lipoproteins prepared with sonicated liposomes (or biological membranes). The following hypothesis may explain the difference: The membrane segment of cytochrome $b_5$, the primary structure of which has recently been elucidated [32—34], contains several charged and polar amino acid residues at the carboxylic end. Owing to their hydrophilic nature, these are presumably not buried in the hydrophobic core of the lipid bilayer [35]. It is equally difficult to imagine that such a hydrophilic sequence can penetrate through a lipid membrane. Therefore the assumption is plausible that in samples made with preformed membranes this part of the polypeptide is on the same side of the membrane as the large, heme-containing part corresponding to trypsin-cytochrome $b_5$. In the case of lipoproteins prepared in the presence of cholate the membrane structure is formed of protein and lipid on removal of the detergent. It is possible that in these cytochrome $b_5$ liposomes the protein spans the lipid bilayer, so that the carboxyl end reaches into the interior of the liposomes. Once the membrane is formed, the hydrophobic core of the bilayer would be impermeable to the sequence near the carboxylic end, therefore the cytochrome is bound irreversibly. Experiments are currently under way to verify this hypothesis. As the NADPH reductase is not transferred from cholate-prepared vesicles either, this reasoning may apply to the reductase, too.

Since endogenous NADPH reductase was also not transferred from microsomes to Golgi membranes and neither cytochrome $b_5$ nor the reductase were transferred from microsomes to liposomes, the linkage of the endogenous proteins to the microsomal membrane must be different from that which anchors the artificially inserted proteins to the microsomes. Catalytically, however, there seems to be no difference between endogenous and artificially incorporated cytochrome $b_5$, at least not in the fatty acid desaturase reaction studied by Strittmatter et al. [36]. In contrast to lipoproteins prepared with sonicated liposomes, the linkage between cytochrome $b_5$ or NADPH reductase and membrane, as seen by the absence of protein transfer, appears to be as stable in microsomes as in cholate-prepared lipoproteins and therefore, might be similar in both cases. But that remains to be proven.

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## References

1 Strittmatter, P. and Velick, S.F. (1956) J. Biol. Chem. 221, 277—286
2 Masters, B.S.S., Baron, J., Taylor, W.E., Issacson, E.L. and LoSpaluto, J. (1971) J. Biol. Chem. 246, 4143—4150
3 Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M.J., Setlow, B. and Redline, R. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4565—4569
4 Hildebrandt, A. and Estabrook, R.W. (1971) Arch. Biochem. Biophys. 143, 66—79
5 Prough, R.A. and Masters, B.S.S. (1974) Arch. Biochem. Biophys. 165, 263—267
6 Duppel, W., Poensgen, J., Ullrich, V. and Dahl, G. (1976), 3rd Symposium on Microsomes and Drug Oxidations, Berlin 1976 (Ullrich, V., Roots, J., Hildebrandt, G., Estabrook, R.W. and Conney, A.H., eds.), pp. 31—38, Pergamon Press, Oxford
7 Enoch, H.G., Fleming, P.J. and Strittmatter, P. (1977) J. Biol. Chem. 252, 565—5660
8 Frommer, U., Ullrich, V. and Staudinger, Hj. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 903—912
9 Holtzman, J.L. and Gillette, J.R. (1967) J. Biol. Chem. 243, 3020—3028
10 Dignam, J.D. and Strobel, H.W. (1975) Biochem. Biophys. Res. Commun. 63, 845—852
11 Holloway, P.W. (1973) Anal. Biochem. 53, 304—308
12 Ozols, J. (1974) Biochemistry 13, 426—434
13 Ehrenreich, J.H., Bergeron, J.J.M., Siekevitz, P. and Palade, G.E. (1973) J. Cell Biol. 59, 45—72
14 Gratzl, M. and Schwab, D. (1976) Cytobiologie 13, 199—210
15 Kaback, H.R. (1971) Methods Enzymol. 22, 253—297
16 Huang, C. (1969) Biochemistry 8, 344—352
17 Poensgen, J. and Ullrich, V. (1977) Biochim. Biophys. Acta 465, 34—45
18 Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322—331
19 Glossmann, H. and Lutz, F. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 1583—1585
20 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
22 Phillips, A.H. and Langdon, R.G. (1962) J. Biol. Chem. 237, 2652—2660
23 Takesue, S. and Omura, T. (1970) J. Biochem. (Tokyo) 67, 259—266
24 Spatz, L. and Strittmatter, P. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1042—1046
25 Ingelman-Sundberg, M. and Glaumann, H. (1978) FEBS Lett. 78, 72—76
26 Pagano, R.E. and Huang, L. (1975) J. Cell Biol. 67, 49—60

27 Roseman, M.A., Holloway, P.W., Calabro, M.A. and Thompson, T.E. (1977) J. Biol. Chem. 252, 4842—4849
28 Holloway, P.W., Katz, J.T., Calabro, M.A. and Riley, L.R., Jr. (1975) Fed. Proc. 34, 642
29 Schulze, H.-U. and Staudinger, Hj. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1675—1680
30 Keenan, T.W. and Morré, D.J. (1970) Biochemistry 9, 19—25
31 De Siervo, A.J. (1969) J. Bacteriol. 89, 1020—1025
32 Ozols, J. and Gerard, C. (1977) J. Biol. Chem. 252, 8549—8553
33 Ozols, J. and Gerard, C. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3725—3729
34 Fleming, P.J., Dailey, H.A., Corcoran, D. and Strittmatter, P. (1978) J. Biol. Chem. 253, 5369—5372
35 Tanford, C. and Reynolds, J.A. (1976) Biochim. Biophys. Acta 457, 133—170
36 Strittmatter, P., Rogers, M.J. and Spatz, L. (1972) J. Biol. Chem. 247, 7188—7194