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Reconversion of Cytochrome *P420* into *P450* and Reactivation of Hydroxylases in Microsomal Membranes Reconstituted by Self-Assembly*

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Membrane vesicles formed by means of dialysis of microsomal proteins and lipids, solubilized by cholate, contained an equal quantity of cytochromes *P420* and *P450*. Addition of albumin and phosphatidylcholine aids reconversion of cytochrome *P420* into cytochrome *P450*. In the presence of albumin the reconstituted membranes contained less lysophosphatidylcholine than those reconstituted without it. Comparative analysis does not reveal marked differences in the phospholipid and protein composition or specific activity and content of NAD(P)H-dependent redox carriers of original and reconstituted membranes. In contrast to original membranes, the reconstituted ones do not possess aniline hydroxylase activity. The NADH-specific dimethylaniline demethylase activity was higher than that of the original membranes. NADPH-dependent demethylase of this substrate could be reconstituted to 50—80% of its initial activity. The reconstituted membranes differ from original membranes by their higher non-enzymatic lipid peroxidase activity. Thus, it appears that dialysis in the presence of albumin and phosphatidylcholine is the most effective mode of microsomal membrane reconstitution.

INTRODUCTION

We demonstrated previously that, as result of self-assembly, membrane vesicles are readily formed[1-3] from microsomal proteins and lipids solubilized with detergent. Membranes reconstituted in such a way possess the enzymatic activity of the starting membranes and contain all microsomal electron carriers. The NAD(P)H-oxidase activities and the peroxidation sistems of lipids[1,2] are easy to reconstitute . The systems of enzymatic hydroxylation could be reactivated to a much lesser degree. Only partial reactivation of cytochrome *P420* to *P450* was observed.

With the aim of increasing the effectiveness of reconstitution, the effect of certain factors was studied, *i. e.* ionic strengths of the medium, pH, bivalent metals ions, phospholipid to protein ratio *etc.*, on the process of self-assembly. The starting and the reconstituted membranes were compared as to their lipid and protein composition and the ultrastructure. Reconversion of cytochrome

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P420 into *P450*, and the rate of hydroxylation and peroxidation were studied as the characteristics of the effectiveness of reconstitution.

EXPERIMENTAL

Materials

Isolation of purified preparation of the ghosts of microsomal vesicle membranes from rat liver was performed as described previously[4].

Solubilization of membrane ghosts. — Ghost fraction with a protein content of 15 mg/ml was treated with sodium cholate in a final concentration of 6% (w/v). Then it was diluted with a Trist-HCl buffer, pH 7.5, which contained 0.2 mM EDTA and 1 mM dithiothreitol (solution TED), to a final concentration of cholate of 1.5% and centrifuged at 150 000 x g for 90 minutes in a VAC-601 centrifuge, rotor 8 × 35. The supernatant (solubilized sample) contained proteins and lipids (Figure 1), that were eluted from a Bio-Gel P-30 column as two distinct peaks. The total yield was 90% of the initial protein of ghost fraction.

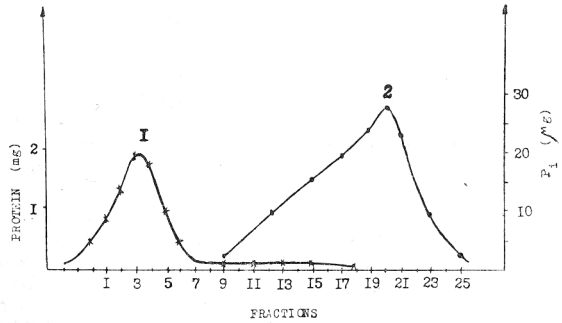

Figure 1. Separation of proteins and lipids in the solubilized sample on a Bio-Gel P-30 column (made by S. Z. Khaitina). Ghosts of microsomal vesicles were treated with sodium cholate 2.6 mg of the detergent per mg of protein and were centrifuged for 90 minutes. Supernatant solution (1 ml) containing 8 mg of protein was applied to a Bio-Gel P-30 column (1.5 × 75 cm). Elution was carried out with 20 mM Tris-HCl buffer, pH 8.1, containing 0.2 mM EDTA, 0.1 M NaCl, 1.5% sodium cholate. Fractions of 1 ml were collected and the protein and phospholipid content was determined. 1 — protein, 2 — phospholipid.

Reconstitution of microsomal membranes. — The solubilized sample (15 ml) containing 2—3 mg of protein per ml, was dialyzed against the solution containing 20 mM Tris-HCl buffer, pH 7.5, 0.2 mM EDTA and 0.1 mM dithiothreitol, for 40 hours at 4 °C the ratio of the volume of the solubilised sample to that of the dialysing solution was 1 to 70. After dialysis the reconstituted material was centrifuged at 150 000 x g for 90 minutes in a VAC-601 centrifuge, rotor 8 × 35. The pellets were suspended in a TED solution.

Methods

The rate of NAD(P)H-dependent *p*-hydroxylation of aniline, *N*-demethylation of dimethylaniline (DMA), the content of cytochromes b_5 and *P450*, and the rate of NADPH- and ascorbate-stimulated peroxidation of lipids were determined as described previously[2].

The protein content was determined by the method of Lowry *et al.*[5] in the presence of 0.1% sodium deoxycholate, with crystalline bovine serum albumin as a standard. The content of phospholipids was measured by the method of Chen *et al.*[6]

A comparative analysis of protein content of the starting and reconstituted membranes was made by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate, according to the method of Fairbanks *et al.*[7]

The material was fixed in an isolation medium by 3—4% glutaraldehyde alone for 24 do 48 hours; or the samples were fixed first by glutaraldehyde, then by 1% OsO_4 for one hour, and then dehydrated in an aqueous solution of acetone and embed-

ded in Epon-812. The sections were contrasted with uranyl acetate and lead acetate[8]. The ultrastructural analysis was carried out in the laboratory of Dr. V. L. Borovyagin (Institute of Biophysics of the USSR Academy of Sciences, Pushchino-on--the Oka).

Analysis of the lipid composition of the membranes was carried out in the laboratory of Dr. L. D. Bergelson (M. M. Shemyakin Institute of Bioorganic Chemistry of the USSR Academy of Sciences, Moscow).

RESULTS

The effect of the duration of dialysis on the effectiveness of reconstitution was studied (Figure 2). This was tested by measuring the ratio of the inactive (absorption maximum at 420 nm) to the active (absorption maximum at 450 nm) forms of cytochromes *P450*. In the starting solubilized sample almost all of the cytochrome was in its inactive form. Reactivation of the haemoprotein on removal of the detergent during the self-assembly was only observed during the first 24 hours of dialysis. Later on, the ratio of the two forms of the cytochrome remains constant. The reconstituted membranes contain a considerable amount of the inactive form of cytochrome *P420*. The total yield of the protein in the reconstituted membranes is not large and is about 25% of all the protein of the solubilized sample.

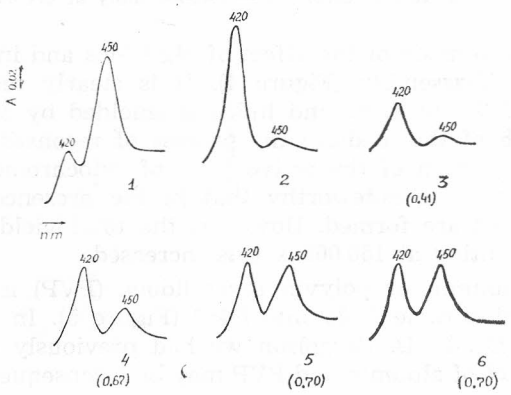

Figure 2. Reconversion of cytochrome *P420* into *P450 versus* the time of dialysis. The solubilized sample (15 ml) was dialyzed against a solution containing 20 mM Tris-HCl buffer, pH—7.5, 0.2 mM EDTA and 0.1 mM dithiothreitol, for 5, 15, 24, and 48 hours at 4 °C. After dialysis, the samples were centrifuged at 150 000 x g for 90 minutes. The sediments were suspended in TED solution and the protein and cytochromes *P420* and *P450* content was determined. Here and in Figures 3—6, in brackets is indicated the yield of membrane protein (mg) per 1 ml of solubilized sample containing 3 mg of protein in 1 ml. 1 — ghosts, 2 — solubilized sample, 3—6 — preparation, obtained after 5, 15, 24, and 48 hours of dialysis, respectively.

Figure 3 shows the effect of glycerol and pH on membrane reconstitution. Glycerol is known to prevent inactivation of cytochrome *P450* to *P420* in microsomal membranes treated with SH-agents, detergents *etc.* and causes reconversion of *P420* into *P450* in the membranes[9,10]. As is obvious from the figure, glicerol did not increase the effectiveness of the reconstitution. The absence of this effect indicates that this reconversion, unlike other known cases of reactivation, cannot be stimulated by polyol alcohols. The increase in the pH of the system from 7.5 to 8.5 did not affect the ratio of cytochrome *P450* and *P420*, in the reconstituted membranes. The decrease in the pH to 6.5 interfered with the reconstitution.

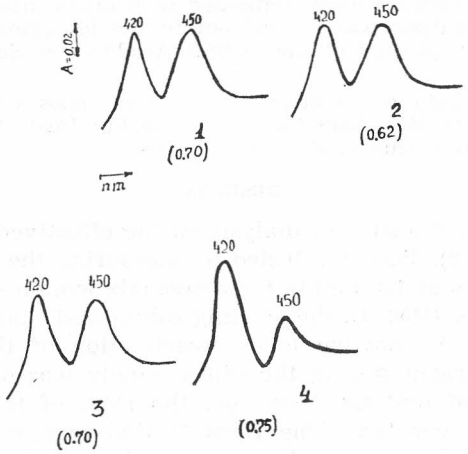

Figure 3. The effect of glycerol and pH on reconversion of cytochrome *P420* into *P450* upon self-assembly. The dialysis was carried out for 40 hours in the conditions of Figure 1. Glycerol was added to solubilized sample and to dialysing solution in a final concentration of 20%. In the experiments on self-assembly at pH 6.5 and pH 8.5 the dialysis was carried out as usual, but pH of the dialysing solution was 6.5 and 8.5, respectively. 1 — self-assembly at pH 7.5, 2 — self-assembly in the presence of 20% glycerol, 3,4 — self-assembly at pH 8.5 and 6.5, respectively.

A study was also made of the effect of Mg^{+2} ions and increase in the ionic strength on the self-assembly (Figure 4). It is clearly seen that when the negative charge of the proteins and lipids is shielded by Mg^{+2} ions and with high ionic strength of the medium the process of reconstitution strongly deteriorates. The proportion of the active form of cytochrome *P450* in the preparations decreases. It is noteworthy that in the presence of Mg^{+2} ions no membrane structures are formed. However, the total yield of the protein in the fraction sedimenting at 150 000 x g is increased.

Addition of albumin or polyvinylpyrrolidone (PVP) is conducive to the reconversion of cytochrome *P420* into *P450* (Figure 5). In collaboration with the laboratory of Dr. L. D. Bergelson we had previously demonstrated that the favourable effect of albumin and PVP may be a consequence of the removal

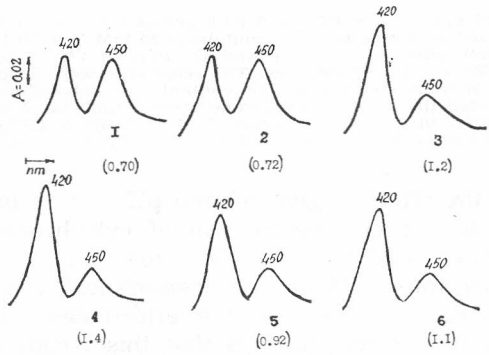

Figure 4. The effect of Mg^{+2} ions and ionic strength of the medium on the effectiveness of reconstitution. Dialysis was carried out for 40 hours in the conditions of Figure 1. $MgCl_2$ at final concentrations of 1 mM, 5 mM and 10 mM, and KCl at final concentrations of 100 mM and 500 mM were added to solubilized sample and the dialysing solution. 1 — self-assembly, 2, 3, 4 — self-assembly in the presence of 1 mM, 5 mM and 10 mM $MgCl_2$, respectively; 5,6 — self-assembly in the presence of 100 mM and 500 mM KCl, respectively.

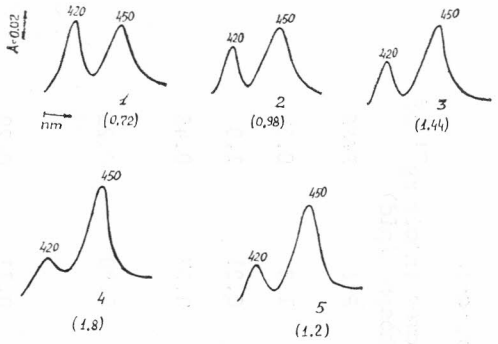

Figure 5. The effect of albumin and PVP on the effectiveness of reconstitution. Albumin 2%, 4%, 10% and PVP 10% was added to 15 ml of solubilized sample. The dialysis was carried cut for 40 hours in the conditions of Figure 1. 1 — self-assembly, 2, 3, 4 — self-assembly in the presence of 2%, 4%, 10% albumin, respectively: 5 — self-assembly in the presence of 10% PVP.

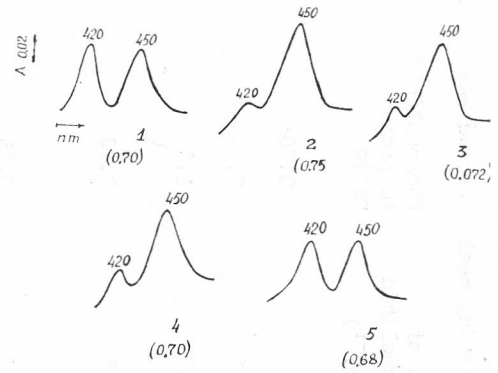

Figure 6. The effect of various phospholipids on the effectiveness of reconstitution. Phosphatidylcholine, microsomal phospholipid, asolectin and phosphatidylethanolamine, 30 mg each, were added to 15 ml of solubilized sample and the dialysis was carried out for 40 hours in the conditions of Figure 1. 1 — self-assembly, 2 — self-assembly in the presence of phosphatidylcholine, 3 — self-assembly in the presence of microsomal phospholipid, 4 — self-assembly in the presence of asolectin, 5 — self-assembly in the presence of phosphatidylethanolamine.

of lysophosphatidylcholine and nonesterified fatty acids from the membranes[11]. The same result was revealed in the study of the effect of phospholipids on the reconstituting system (Figure 6). The ability to enhance the reconversion of cytochrome *P420* into *P450* was displayed by all the preparations of phospholipids containing phosphatidylcholine. Phosphatidylethanolamine proved ineffective in the reconstitution of the membrane system. Thus, phosphatidylcholine is indispensable for the native structure of cytochrome *P450* to be maintained in the membrane.

The chemical composition of the starting and reconstituted membranes is shown in Table I. It is obvious from the results obtained that the yield of protein in the reconstituted membranes amounts to not more than 25% of the starting protein. The addition of albumin and PVP increases the yield of the protein in the reconstituted membranes. At the same time, the yield of phospholipids in the reconstituted membranes is much higher, 40%. The phospholipid to protein ratio in the membranes reconstituted by self-assembly is higher than in the initial membranes.

TABLE I

Chemical composition of the starving membranes and reconstituted membranes

Fraction or procedure	Yield of protein ($mg \cdot ml^{-1}$)	Yield of phospholipid ($mg \cdot ml^{-1}$)	Phospholipid to protein ratio	Cytochromes (nmole mg^{-1} of phospholipid)		
				b_5	*P450*	*P420*
Ghosts			0.70	0.87	1.3	0.34
Solubilized prep.	3.0	1.6	0.53	0.90	0.21	1.3
Self-assembly	0.70	0.70	1.0	0.78	0.37	0.45
Self-assembly in the presence of 10% albumin	1.8	0.6	0.33	1.2	0.80	0.21
Self-assembly in the presence of 10% PVP	1.2	0.8	0.75	0.76	0.71	0.20

Measurement of the electron carriers showed that the membranes reconstituted by self-assembly contain the same quantity of cytochrome b_5 as the starting membranes. Addition of albumin increases the content of cytochrome b_5 in the reconstituted membrane. At the same time, the content of cytochrome *P450* in the reconstituted membranes is much lower than in the starting ones. The addition of albumin and PVP to the system to be reconstituted increases the content of cytochrome *P450* and facilitates its formation from the inactive *P420*.

The ultrastructural analysis made in the laboratory of V. L. Borovyagin showed that removal of the detergent during dialysis entails formation of membrane structures (vesicles), as revealed after aldehyde plus osmium fixation. The membrane structures fixed with aldehyde alone are seen as very long structures, whereas vesicles characteristic of aldehyde plus osmium fixation completely disintegrate. The starting membranes have a three-layer structure with both fixation procedures. In the presence of albumin and PVP, the three--layer organization of membrane vesicles is retained if the preparations are fixed with aldehyde plus osmium. Membrane vesicles cannot be revealed by aldehyde fixation alone. All the material is then represented by unclosed fragments of three-layer membranes. Observation that the structure of reconstituted membranes is retained after fixation by glutar aldehyde and osmium tetraoxide, and that membrane vesicles are almost completely disintegrated after fixation by glutar aldehyde alone followed by dehydratation by organic solvents, indicate that the membranes formed contain a bimolecular layer of lipid molecules in the interphase regions in which protein components are localized in a discontinuous fashion[8].

The structure of ghost membranes is retained with aldehyde fixation only. This means that the starting and reconstituted membranes have different molecular organization.

An electrophoretic analysis of the protein composition of the starting and reconstituted membranes showed that, as a result of self assembly, membranes are formed whose protein composition resembles the starting ghost membranes. The reconstituted membranes contained all the fractions except the one that had the lowest mobility in polyacrylamide gel (fraction 1 of a molecular weight of 200 000) and one rapidly moving fraction (fraction 18 of a molecular weight of 23 000). The proteins of a molecular weight of 80 000 (fraction 7) are hardly ever incorporated into the reconstituted membranes. Addition of albumin and PVP to the system to be reconstituted did not improve the incorporation of these proteins into the membranes.

An analysis of the lipid and phospholipid composition of the reconstituted membranes[11] showed that the latter differ from the starting membranes only having a higher content of lysolecithin (Table II). Addition of albumin and PVP decreases the content of lysolecithin in the reconstituted membranes, most probably due to its binding with albumin. The decrease in the content of lysolecithin in the membranes may enhance reactivation of cytochrome *P420* into *P450*. Lysolecithin is known to induce inactivation of native haemoprotein[12].

In the reconstituted membranes, the rates of oxidation of DMA and aniline and the activity of the peroxidation systems of unsaturated fatty acids were measured (Table III). It turned out that the membranes reconstituted by self--assembly possess a low ability to utilize NADPH as a cosubstrate in *N*-demethylation of DMA (28% of the initial level). NADH in this reaction is used

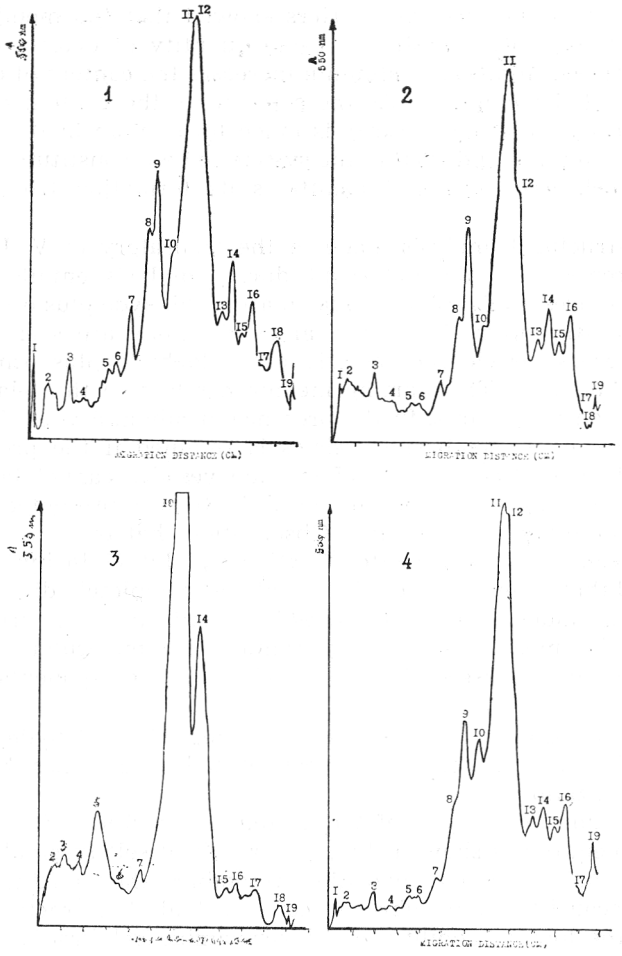

Figure 7. Electrophoretic analysis of the protein composition of the initial and reconstituted membranes (made by V. O. Zhihareva). The samples with a protein content of 1—1.5 mg were added to 1% sodium dodecylsulphate, dissolved in 10% sucrose containing 10 mM Tris-HCl buffer, pH 8.0, 0.1 mM EDTA and 40 mM dithiothreitol. After incubation at 37 °C for 30 minutes the samples were applied to 5.6% polyacrilamide gel (6 mm × 100 mm). Electrophoresis was carried out at a constant voltage of 5 V/cm. The time of distillation in such conditions was 3.5 hours. After electrophoresis the gel was placed in 10% trichloracetic acid. The gel was stained with Coomassi blue and scanned at 550 nm in a Gilford spectrophotometer: 1 — ghosts, 2 — self-assembly, 3 — self-assembly in the presence of 10% albumin, 4 — self-assembly in the presence of 10% PVP.

more effectively as a cosubstrate (60% of the initial activity). The same relationship is observed in the hydroxylation of aniline. The enzymatic peroxidation system is reconstituted in such membranes by 60%, whereas nonenzymatic peroxidation in the reconstituted membranes is 1.5 times as high as the initial rate. Thus the functional activity of the membranes reconstituted by self-assembly differs considerably from that of the starting membranes. Addition of albumin or PVP to the system to be reconstituted increases the effectiveness of the reconstitution of NADPH-dependent *N*-demethylation of DMA (80% of the initial level). The rate of NADH-dependent *N*-demethylation of DMA in such

TABLE II

Phospholipid composition of microsomal vesicle ghosts and reconstituted membranes (per cent of phosphorus)

Phospholipids	Ghosts	Reconstituted membranes		
		Self-assembly	Self-assembly in the presence of 10% albumin	Self-assembly in the presence of 10% PVP
Phosphatidyl-ethanolamine	22.2	21.2	19.8	22.2
Phosphatidyl-choline	54.7	51.4	58.8	59.0
Phosphatidyl-serine + phos-phatidylinositol	15.5	12.5	13.7	11.0
Sphingomyelin	5.6	6.8	5.3	5.1
Lysolecithin	2.0	8.1	2.4	2.7

TABLE III

The effect of albumin and PVP on the reconstitution of hydroxylation and peroxidation systems

Fraction or procedure	*N*-Demethylation of DMA		*p*-Hydroxylation of aniline		Lipid peroxidation	
	NADPH	NADH	NADPH	NADH	NADPH-dependent	Ascorbate-dependent
Ghosts	12.0	2.0	1.54	0.44	4.3	4.1
Solubilized prep.	0	0	0	0	0	0
Self-assembly	3.5	1.2	0.15	0.077	2.5	6.0
Self-assembly in the presence of 10% albumin	8.8	4.0	0.094	0.070	5.3	11.3
Self-assembly in the presence of 10% PVP	8.8	2.7	0.18	0.074	5.0	14.7

Note. The demethylation of DMA, *p*-hydroxylation of aniline and lipid peroxidation were calculated as nmol of formaldehyde, nmol of *p*-aminophenol and nmol of malonic dialdehyde formed per mg phospholipid per minute, respectively.

membranes is 1.5 to 2 times as high as that in the starting membranes. The rate of enzymatic peroxidation was 1.2 times and that of nonenzymatic peroxidation 2.5 to 3 times as high as in the starting membranes. At the same time, aniline *p*-hydroxylation activity under the same conditions amounted to only 5 to 10% of the initial level. The results obtained allow the conclusion to be made that the reconstituted membranes differ from the initial ones by their hydroxylase activity and activity of the peroxidation systems.

DISCUSSION

The study we carried out has shown that, as a result of self-assembly, membrane vesicles are formed containing all microsomal electron carriers[2]. At the same time, 50% of the cytochrome *P450* is found in its inactive form, *P420*. In the starting solubilized system almost all of cytochrome *P450* is in the form of *P420*. The mechanism of reconversion of cytochrome *P420* to *P450* is still obscure. It is likely that the reactivation is a consequence of incorporation into the lipid phase of the newly formed membranes. The mechanism of reconversion differs from the well-known mechanism of reactivation of cytochrome *P420* into *P450* in the presence of polyol alcohols. Addition of glycerol in the system to be reconstituted does not affect the degree of reaction of cytochrome *P450* (Figure 3). To enhance the effectiveness of self-assembly, use may be made of albumin, PVP and phosphatidylcholine. Membranes reconstituted in the presence of these compounds contain almost all of the cytochrome *P450* in the active form and possess a high hydroxylase activity. One of the possible mechanisms by which these compounds produce their effect is to decrease the concentration of lysolecithin in the reconstituted membranes. The newly formed membranes have a similar protein and lipid composition to that of the initial ones. At the same time, not all the protein fractions of the ghosts can be found in the reconstituted membranes. Therefore, the task of our further experimental work is to incorporate these proteins into reconstituted membranes.

It may be inferred from the results obtained that, as a result of self-assembly, the membranes obtained are in many respects similar to the initial membranes of microsomal vesicles. However the efficiency of the self-assembly is not high, *i. e.* not more than 30% of the total protein of the solubilized sample is found in the reconstituted membranes. This allows the suggestion to be made that in the cell there are certain factors capable of regulating the process of *de novo* formation of the membranes.

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SAŽETAK

Povrat citokroma *P420* u *P450* i reaktivacija hidroksilaza mikrosomnih membrana rekonstituiranih *in vitro*

A. I. Archakov, G. I. Bachmanova i I. P. Kanaeva

Membranske vesikule, nastale dijalizom proteina i lipida iz mikrosoma solubiliziranih prvotno kolatom, sadržavale su podjednako citokroma *P420* i *P450*. Dodatak albumina i fosfatidilkolina potiče pretvorbu forme *P420* u *P450*. U prisustvu albumina rekonstituirane membrane imale su manje lizofosfatidilkolina. Usporedna analiza nije pokazala velike razlike u sastavu između originalnih i rekonstituiranih membrana na fosfolipidima i proteinima, niti u specifičnoj aktivnosti i količini NAD(P)H-zavisnim redoks-nosiocima. Protivno originalnim membranama one rekonstituirane nisu imale aktivnosti anilinske hidroksilaze, dok im je aktivnost NADH-zavisne dimetilanilin-demetilaze bila pojačana. NADPH-zavisno demetiliranje tog substrata bilo je 50—80% izvorne aktivnosti. Rekonstituirane se membrane razlikuju od originalnih i po povećanoj ne-enzimskoj peroksidaciji lipida. Zaključuje se da je dijaliza u prisustvu albumina i fosfatidilkolina najefikasniji način rekonstitucije mikrosomskih membrana.

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