Sequential Solubilization of Microsomal Mixed Function Oxidases*

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SUMMARY

Addition of increasing levels of sodium deoxycholate to fixed quantities of liver microsomes from noninduced mice reveals a sequential release, in three discrete steps, of certain elements of the mixed function oxidase system.

Only one of these stages influences aniline and naphthalene oxidase activity, suggesting the possibility that sequential release is related to differentiation of function.

The cytochrome P-450 and the NADPH₂-dependent cytochrome c reductase (EC 1.6.2.3) components can be separated by ammonium sulfate precipitation and reconstituted to form a system active against naphthalene.

The partial removal of sodium deoxycholate from solubilized cytochrome P-450 by the addition of Dowex 1X 2-400 results in a decrease of contaminant cytochrome P-420 with a concomitant increase in the amount of cytochrome P-450, a phenomenon which suggests renaturation of cytochrome P-420 into cytochrome P-450.

Studies in several laboratories have led to the solubilization and resolution of the cytochrome P-450 containing enzyme system of hepatic (1-11) adrenal cortex (12) and kidney cortex microsomes (13), as well as systems found in bacteria, especially the camphor grown Pseudomonas putida strain PpG786 (14-19).

The procedures used, generally involve treatment of microsomal suspensions with either sodium deoxycholate (1-6), Triton X-100 (13), or Triton N-101 (11), followed by DEAE-cellulose column chromatography (1-6), ammonium sulfate precipitation, and DEAE-cellulose chromatography (6, 9, 11). In some cases, ammonium sulfate precipitation is preceded by sonication (6) or followed by alumina gel Cy-pretreatment (11). Cytochrome b₅ has been eliminated by treatment with Bacillus subtilis protease followed by ammonium sulfate precipitation (7). The problem is simplified in the case of Pseudomonas, since the oxidase system is already soluble (14). However, no detailed information is at present available on the kinetics of the release of microsomal electron transfer system components during solubilization, which would seem essential for a direct approach to this problem. The present study reports the solubilization of mouse liver microsomal mixed function oxidase system with increasing amounts of sodium deoxycholate which brings about a sequential release in three discrete steps of some of the system's components.

EXPERIMENTAL PROCEDURE

Liver microsomes were obtained from 8-week-old Swiss Webster mice. The animals were killed by decapitation, the livers removed, cut into small pieces that were repeatedly washed by decantation with 0.1 M KCl-0.1 M sodium citrate buffer, pH 7.7 (Medium A) to eliminate as much hemoglobin as possible, and then homogenized with 3 volumes of Medium A using a glass homogenizer fitted with a Teflon pestle. The homogenate was filtered through three layers of cheesecloth, and the filtrate centrifuged twice at 15,000 × g × 15 min to remove nuclei, cellular debris, and mitochondria. Two-thirds of the supernatant fluid were then centrifuged at 55,000 rpm for 60 min in the 60 Ti rotor of a L2-65B Spinco centrifuge. All the above operations were conducted at 2-4°. The microsomal pellet was thrice washed with the original volume of Medium A by centrifuging at 55,000 rpm as above. The washed microsomal pellet was finally resuspended in Medium A containing 30% glycerol (w/v, Medium B) and the suspension was again centrifuged at 55,000 rpm × 5 hours. The resulting pellet was resuspended in Medium B to yield a protein concentration of 25 mg per ml, dispersed into 0.1-ml aliquots, quickly frozen under liquid nitrogen and stored at −80° until used.

The results reported here were obtained from a single preparation of microsomes obtained from 50 mice. Microsomes can be stored at −80° without any loss in cytochrome P-450, NADPH₂-dependent cytochrome c reductase, and hydroxylating activity.

Solubilization of Microsomes—Solubilization was carried out as follows. Weighed amounts of Medium B containing 10% sodium deoxycholate (Medium C) were added to 0.1-ml aliquots of microsomal suspensions to obtain sodium deoxycholate concentrations varying from 0.1 to 3%. The volume of Medium C added was between 1 to 30 µl. The solubilized preparations were diluted with 1.7 ml of Medium B and the resulting mixture was centrifuged at 50,000 rpm × 1 hour in the 60 Ti rotor as above but using 2.0-ml centrifuge tubes.

Fractionation of microsomal components was done as follows. Microsomes, 2.2 ml, in Medium B were diluted to 39.6 ml with the same medium, and then 2.1 ml of Medium C added to give a final sodium deoxycholate concentration of 0.5%. The mix-
The pellet was removed with a porcelain spatula, the pH of the dropwise addition of cold 1.0 M supernatant fluid when necessary adjusted to pH 7.7 by the addition of NaOH, and the concentration of ammonium sulfate added raised to 60% as above. The supernatant fluid was then centrifuged at 55,000 rpm for 1 hour in the D-2 Ti rotor. The resulting precipitate was dissolved in 1.0 M NaOH, and the concentration was adjusted to pH 7.7 by the dropwise addition of 0.5 NaOH. The precipitate was redissolved in Medium B and the supernatant fluids were centrifuged at 20,000 rpm for 5 min and the supernatant fraction was added to the reaction mixture. The precipitate was dissolved in Medium B containing 0.05% sodium deoxycholate. To 1.8 ml of a solubilized preparation, 25 µl of 10% sodium deoxycholate were added, followed by a minimal amount of saturated sucrose for application of the sample under buffer. The sample was divided in half and the elution volume for cytochrome P-450 and NADPH-cytochrome c reductase determined twice. Duplicate determinations of the void volume, which corresponds to NADPH-cytochrome c reductase, were reprecipitated by the addition of 60% ammonium sulfate. The pellet was redissolved in Medium C and stored at -80°C until further use.

The floating pellet which contained cytochrome P-450 was dissolved in a minimum volume of Medium C, dialyzed against the same medium for 3 hours, and then diluted to a final volume of 2.0 ml as above. The material was stored at -80°C. It should be pointed out that enzyme assays were usually carried out within 12 hours of storage.

### Spectral Determinations

**Cytochrome b5** was determined from the difference spectra between the oxidized and the reduced forms, as described by Capdevila et al. (20) and using an extinction coefficient of 163 cm⁻¹ M⁻¹ for the difference between 424 and 409 nm (21). Cytochrome P-450 was estimated according to Omura and Sato (22) as described by Morello et al. (23). An extinction coefficient of 91 cm⁻¹ M⁻¹ for the difference between 490 and 450 nm was used for this purpose (22). The NADPH-dependent cytochrome c reductase (EC 1.6.2.3) was determined by following the reduction of cytochrome c at 550 nm as described by Masters et al. (24) and Capdevila et al. (20, 25).

**Hydroxylation Activity**—The hydroxylation of aniline to p-aminophenol and of naphthalene to 1,2-dihydro-1,2-dihydroxynaphthalene and 1 naphthol were followed using the reaction mixtures of Morello et al. (23). The products of naphthalene hydroxylation were determined as previously reported (20, 23) while p-aminophenol was estimated by the procedure described by Kato and Gillette (26).

**Removal of Sodium Deoxycholate**—To reduce the sodium deoxycholate concentration from enzyme suspensions, Dowex 1X 2-400, previously washed with 20 volumes of deionized water by filtration through a Buchner funnel, was added at the ratio of 0.1 g of Dowex per ml of enzyme. The mixture was centrifuged at 2000 g for 5 min and the supernatant fraction was aspirated off and saved. The precipitate was washed twice with 0.25 volumes of Medium B and the supernatant fluids were combined and stored at -80°C. No sodium deoxycholate could be detected in these supernatant fluids by lowering the pH to 6.8. To determine the amount of sodium deoxycholate that could remain bound to cytochrome P-450, fractionation of the microsomal components as described above was carried out with sodium deoxycholate containing [³H]labeled sodium deoxycholate (specific activity, 40 µCi per mg). Aliquots were taken during all fractionation steps, added to scintillation vials and the radioactivity determined as indicated below. Under these conditions, the sodium deoxycholate bound to the cytochrome P-450 preparation corresponds to 0.26 µmole per mg of protein (0.81 µmole per nmole of cytochrome P-450). The final concentration of sodium deoxycholate in the enzyme solution was 0.05%. About 88% of the original sodium deoxycholate was removed by the ammonium sulfate precipitation (49%) and the dialysis step (39%).

### Molecular Weight

Molecular weights of the fractionated microsomal components were determined by use of a Sephadex G-200 column (1 x 30 cm), equilibrated with Medium D containing 0.05% sodium deoxycholate. To 1.8 ml of a solubilized preparation, 25 µl of 10% sodium deoxycholate were added, followed by a minimal amount of saturated sucrose for application of the sample under buffer. The sample was divided in half and the elution volume for cytochrome P-450 and NADPH-cytochrome c reductase determined twice. Duplicate determinations of the void volume with blue dextran were also done.

### Analytical Procedures and Reagents

**Protein** was determined according to Lowry et al. (27). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP, NADPH, Dowex 1X 2-400, and crystallized bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium deoxycholate was purchased from Schwartz-Mann, New York, N. Y. Uniformly labeled [³H]deoxycholic acid (specific activity, 1.2 Ci per mg) was purchased from Mallinkrodt, St. Louis, Mo. The rest of the chemicals used were of the highest purity commercially available. All spectral determinations were made at 22°C in a Unicam SP-1800 spectrophotometer, using silica cells of 1-cm light path. Radioactivity was measured as previously described (20) using Bray's scintillation mixture (28).

### RESULTS

The patterns of microsomal sedimentation during solubilization were followed at sodium deoxycholate concentrations ranging from 0.1 to 3.0 with 0.1% increments each time. Both the floating pellet and the supernatant fluid were analyzed at each sodium deoxycholate concentration for NADPH-dependent cytochrome c reductase, cytochrome P-450, and total protein. Either Na₂SO₄ or NADPH was routinely used for reduction of cytochrome b₅, since no apparent differences were found when Na₂SO₄ was replaced by NADPH. Fig. 1 shows the sedimentation behavior of cytochromes P-450 and b₅ at the most significant sodium deoxycholate concentrations. At a concentration of 0.1% sodium deoxycholate, most of the hemoproteins are found in the pellet, and only minute amounts, especially of cytochrome P-450, are observed in the supernatant fluid. When the concentration of sodium deoxycholate is raised to 0.5%, a sharp differentiation in the behavior of the cytochromes P-450 and b₅ is observed. About 50% of the cytochrome P-450 becomes soluble while most of the cytochrome b₅ remains particulate. Differences in solubility patterns seem to stabilize at this sodium deoxycholate concentration of 1.3%, with 50% of each hemoprotein being soluble. Another phenomenon now becomes apparent, 50% of cytochrome P-450 is solubilized with 0.5% sodium deoxycholate, but an increase of sodium deoxycholate to 0.8% dissolves the remaining 50% of cytochrome P-450. A 0.5% concentration of sodium deoxycholate is insufficient to solubilize significant amounts of cytochrome b₅, while an increase in sodium deoxycholate concentration to 0.8% solubilized about 50%. This nonlinearity is observed until the system is almost completely solubilized. Residual amounts of the hemoproteins are still particulate at sodium deoxycholate concentrations of 3.0%.
The nature of the above phenomenon is clearly shown in a point by point analysis (Fig. 2). Three discrete levels of release are apparent for cytochrome P-450. Firstly, a sharp release of approximately 50% of the hemoprotein between 0.1 and 0.5% sodium deoxycholate concentrations; secondly, a small rate of release between 0.5 and 0.8% sodium deoxycholate concentrations; and thirdly, another sharp rise in the solubilization of the hemoprotein at sodium deoxycholate concentrations between 0.9 and 1.3% with a leveling off above the latter concentration. While no attempt has been made to demonstrate an increase in the amount of soluble cytochrome P-450 beyond 1.3% sodium deoxycholate, it would appear that the remaining particulate cytochrome P-450 may be either solubilized or denatured at a slow rate at sodium deoxycholate concentrations ranging from 1.3% to 3.0% which suggests a third type of cytochrome P-450 release.

As far as NADPH₂-dependent cytochrome c reductase is concerned, three steps are evident, two of which coincide with the solubilization steps of cytochrome P-450 while a third stage of release is present at sodium deoxycholate concentrations between 2.1 and 3% (Fig. 2).

These areas of release also are apparent in the case of cytochrome b₅. Similar results were obtained when the oxidized versus the reduced difference spectra were determined with either NADPH₂ or Na₂S₂O₄ (Fig. 2). The pattern of solubilization of cytochrome b₅ in the 0 to 0.5% sodium deoxycholate region is difficult to interpret. Since both the NADPH₂ and the Na₂S₂O₄ patterns are similar, it would appear that the changes observed may not be related to the reduction of hemoprotein, but to a phenomenon of reversible solubilization. However, it should be pointed out that the data are rather complex, and may reflect two combined effects, that is a dissociation by sodium deoxycholate followed by a reassociation on dilution. In experiments not shown here, we have observed that it is not possible to start with the 0.5% sodium deoxycholate fraction and then obtain a curve with the same shape as described in Fig. 2 when sodium deoxycholate is increased.

The solubilization patterns of protein are similar to those of cytochrome P-450, and only diverge from the patterns observed for cytochrome b₅ and the NADPH₂-dependent cytochrome c reductase in the last stage, which suggests a phenomenon of differential solubility in this region. Approximately 30% of the protein is not solubilized with sodium deoxycholate concentrations of 3%, which may imply that the majority of the membrane system is tied in with the solubilization of the hydroxylation system. These observations support the previous results concerning stepwise release of microsomal components and suggest that the phenomenon involves differential solubility of groups of enzymes. These results may be explained by either a three step solubilization of a single protein species or as the patterns of solubility for three differentially associated protein groups.

The effects of increasing concentrations of sodium deoxycholate on the hydroxylation of type I and II substrates (20, 29) are shown in Fig. 3. At sodium deoxycholate concentrations up to 0.5% the largest decrease in activity for aniline and naphthalene hydroxylation occurs. The hydroxylation activity remains fairly constant at concentrations between 0.9 and 1.3% sodium deoxycholate followed by a slight decrease in the last stage of release, 2.2 to 3.0% sodium deoxycholate. Since the hydroxylation activity is mainly affected at sodium deoxycholate concentrations between 0 and 0.5% and not on further solubilization, it appears that the hydroxylation activity is localized in the supernatant fraction produced at this release level (Fig. 3). Examination of the activity of the supernatant fluid and the pellet separately support this explanation. The loss in activity

![Diagram](http://www.jbc.org/)

**Fig. 1.** Sequential solubilization of cytochromes b₅ and P-450. The top of each frame represents the pellet and the bottom the supernatant fraction at their respective sodium deoxycholate concentrations. The left-hand member represents cytochrome b₅ as determined by NADPH₂ reduction, the right one cytochrome P-450. All spectra correspond to undiluted samples. Percentage sodium deoxycholate relative to initial microsomal volume.
FIG. 2. Point by point analysis of sequential solubilization. All Y axis measurements are in units per ml of final volume as in the solubilization protocol. Percent sodium deoxycholate relates to initial microsomal volume. Pellets are in closed symbols, supernatants open. Bottom frame left represents cytochrome b$_5$ determined from the oxidized versus the reduced difference spectra using NADPH$_5$, middle with Na$_2$S$_2$O$_4$. Standard errors are indicated and where not shown are within the area of the symbol used. In cases where adjacent points are close by, only 1/2 the standard error is indicated on one side of the mean. Cytochrome P-450 and NADPH$_5$-dependent cytochrome c reductase measurements represent an average of five determinations for each individual point. Each point is represented by an average of four determinations for NADPH$_5$-dependent cytochrome c reductase activity, cytochrome b$_5$ determined by NADPH$_5$ reduction, and protein, and an average of three determinations for cytochrome b$_5$ determined by Na$_2$S$_2$O$_4$ reduction.
Fig. 3. Effect of solubilization on type I and II hydroxylations, expressed as nanomoles of end product. Top, hydroxylation of aniline and naphthalene prior to separation of supernatant and pellet fractions by sedimentation, no Dowex added. Middle, allocation of activity to supernatant and pellet fractions after centrifugation, no Dowex added. Bottom, effect of Dowex on activity spectrum of separated fractions. All determinations are made with an initial volume of 0.68 ml of appropriate enzyme solution and hence represent in the top frame fixed levels of enzyme and in all other levels that increase in the supernatant and decrease in the pellet with increasing sodium deoxycholate concentration. The top frame represents an average of four determinations for each point, the bottom frame 2.

The lack of complete agreement between results from composite preparations, that is, the fall off in activity in the supernatant fluid as opposed to rather stable levels without separation, may be explained, at least in part by the cooperative effects between soluble and particulate fractions. This is suggested from the results obtained when sodium deoxycholate is almost completely removed from the supernatant fluid and the pellet by the addition of Dowex 1X 2-400. Partial removal of sodium deoxycholate results in a reversal of the loss of activity in the supernatant fluid. Dowex 1X 2-400 itself does not apparently affect the hydroxylation activity since it neither changes the pattern of loss of activity of the pellet nor the activity of the 0.5% sodium deoxycholate supernatant fluid. These results suggest that removal of sodium deoxycholate may correlate with the possibility of a reassociation and that reversal of the loss of activity of the supernatant fluid in the presence of increasing sodium deoxycholate concentrations removed by Dowex may initiate a compensatory phenomenon for the increased solubility of this fraction. In this connection, the increase in activity in the final stages of solubilization suggests that the last step of release either enhances the activity of the 0.5% sodium deoxycholate fraction or may itself be involved in the activity toward aniline and naphthalene. It is interesting to note that the cytochrome P-450 fraction present in the floating pellet was contaminated with small amounts of cytochrome P-420. Part of the latter could be precipitated during centrifugation at 55,000 rpm × 60 min. Furthermore, when Dowex was added to the
dialyzed preparation to remove sodium deoxycholate, most of the remaining cytochrome P-450 disappears with a concomitant increase in the amount of cytochrome P-450, which suggests a renaturation phenomenon.

The similarities observed for the hydroxylating activity of the supernatant fluid toward aniline and naphthalene is rather surprising since naphthalene is a type I substrate (20, 23, 25, 29), while aniline is a type II one (30). The solubilization procedures used in several laboratories (1-10) result in a system capable of hydroxylating type I substrates (28), while the reverse occurs with the procedure used by Fujita and Mannering (11) whose solubilized preparations preferentially hydroxylate type II substrates. However, it should be pointed out that our results have been obtained with microsomal fractions derived from previously untreated animals, while those reported from other laboratories (1-11, 31) correspond to animals induced with either 3-methylcholanthrene or phenobarbital. Nevertheless, it has been recently reported (31) that solubilised cytochrome P-450 from untreated and phenobarbital-treated rats as well as the cytochrome P-448 fraction from 3-methylcholanthrene-induced animals are all active for aniline hydroxylation, but the cytochrome P-448 fraction is consistently more active than the cytochrome P-450 one.

Our results suggest that differences in activity toward either type I and type II substrates are the result of conformational changes occurring on separation alone, or to differential denaturation of one of the system components by chemical treatment. It is interesting to point out that treatment of mammalian microsomal fractions with phospholipase C (32) results in loss of activity toward type I substrates while hydroxylation of aniline is only slightly decreased. Although sufficient material was not available to test the effects of fractionation on the activity toward aniline, the addition of sodium deoxycholate at levels which do not result in significant losses of naphthalene hydroxylation activity, facilitates an almost complete separation of cytochrome P-450 from NADPH-dependent cytochrome c reductase by ammonium sulfate fractionation. Cytochrome P-450 is found as a floating pellet at 40% ammonium sulfate concentration with a final yield of 22%, while the reductase remains in the supernatant fluid. The latter enzyme is unstable at this ammonium sulfate concentration, but it can be stabilized by precipitation at 60% ammonium sulfate concentration followed by removal of the salt by Sephadex G-25 filtration, with a yield of about 20%. V/Vo values separately obtained for the NADPH-dependent cytochrome c reductase fraction and cytochrome P-450 by Sephadex G-200 column chromatography were about 1.0. While no claim is made that the two fractions, i.e. the cytochrome P-450 and the NADPH-dependent cytochrome c reductase are pure, they are sufficiently separated for reconstitution studies (Table 1). The NADPH-dependent cytochrome c reductase alone did not show any hydroxylation of naphthalene while cytochrome P-450 had some activity suggesting some cross-contamination with the reductase. The activity increased somewhat when both cytochrome P-450 and the reductase were combined (Experiment 3). However, when the amount of reductase was doubled while maintaining the concentration of cytochrome P-450 (Experiment 4), the activity was greatly enhanced. This enhancement may be visualized as due to the increase in the amount of reductase added, suggesting that the limiting step of the reaction is the reduction of cytochrome P-450. It is possible that the addition of higher amounts of reductase involve increasing the phospholipid level in the reaction mixture. The latter have been shown to be required for the reduction of cytochrome P-450 in rat liver microsomal preparation (33, 34). It should be pointed out that when cytochrome P-450 was mixed with the NADPH-dependent cytochrome c reductase, the absolute spectrum of cytochrome P-450 apparently showed an increase in absorbance at 416 nm suggesting that reconstitution of the system in its usual proportion could be associated with an increase in the extinction coefficient of the hemoprotein. This phenomenon appeared to be rather surprising because it would indicate either a previously undemonstrated specificity con-

### Table 1

**Reconstitution of solubilized mouse liver microsomal mixed function oxidase system**

Naphthalene hydroxylation was followed by measuring the sum of 1,2-dihydro-1,2-dihydroxy-naphthalene and 1-naphthol produced. The reaction mixtures contained in a final volume of 1.0 ml, the following: MgCl₂, 0.5 μmoles; NADP, 0.6 μ mole; glucose 6-phosphate, 15 μmoles; glucose 6-phosphate dehydrogenase, 1 unit; enzyme fraction, 0.5 ml. The reaction was started by the addition of 5 μmoles of naphthalene in 25 μl of ethanol, and incubation was carried out at 37° for 10 min. The reaction was stopped by the addition of 0.1 ml of concentrated HCl, the tubes were then heated in a boiling water bath for 10 min to convert the diol into 1-naphthol. The samples were extracted with 3 ml of ethyl acetate, and the 1-naphthol determined in the ethyl acetate layer with 2,6-dichloroquinonechloroimide. Zero time values were separately obtained for the NADPH-dependent cytochrome c reductase fraction and cytochrome P-450. Zero time values separately obtained for the NADPH-dependent cytochrome c reductase fraction and cytochrome P-450. Zero time values separately obtained for the NADPH-dependent cytochrome c reductase fraction and cytochrome P-450.

| Experiment | Fraction | Protein | Cytochrome P-450 | NADPH-dependent cytochrome c reductase | Naphthalene hydroxylated | Naphthalene hydroxylated |
|------------|----------|---------|-----------------|--------------------------------------|-------------------------|-------------------------|
| 1          | NADPH-dependent cytochrome c reductase | 0.09    | 0.000           | 0.0                                  | 0.0                     | 0.0                     |
| 2          | Cytochrome P-450              | 0.38    | 0.29            | 0.0                                  | 0.16                    | 0.02                    |
| 3          | Cytochrome P-450 + NADPH-dependent cytochrome c reductase (0.009 units) | 0.47    | 0.29            | 0.0                                  | 0.32                    | 0.52                    |
| 4          | Cytochrome P-450 + NADPH-dependent cytochrome c reductase (0.015 units) | 0.06    | 0.29            | 0.015                                | 0.93                    | 1.8                     |
| 5          | 0.5% sodium deoxycholate supernatant | 0.56    | 0.29            | 0.000                                | 0.56                    | 0.73                    |
| 6          | Microsomes                     | 0.78    | 0.57            | 0.062                                | 1.11                    | 1.53                    |
tributed by the reductase itself, or the presence in the reductase fraction of other element undetected at the moment. As a result it was decided to further investigate this point. Microsomes were fractionated as indicated under "Experimental Procedure," with the difference that they were prepared in 0.1 M "Tris" buffer, pH 7.7 instead of Medium A. The rest of the procedure was unchanged. When the cytochrome P-450 and NADPH-dependent cytochrome c reductase fractions were combined, the CO-difference spectra did not show any increase in the amount of hemoprotein. Analysis of the reductase fraction indicated a variable but small contamination with cytochrome b₅ while the cytochrome P-450 fraction also contained some cytochrome P-420. It is obvious that when the absolute spectrum is done in the reconstituted system, any cytochrome b₅ present in the reductase fraction or cytochrome P-420 contaminating the cytochrome P-450 fraction will not be balanced in the sample and reference cuvettes, and as a result an increase in absorbance at 416 nm of cytochrome P-450 may be apparent since oxidized cytochromes b₅ and P-420 absorb in the same region.

**DISCUSSION**

While stepwise solubilization is an important phenomenon in itself, as a parameter for separation of the microsomal system, it carries the possibility of broader implications.

If the specificity of naphthalene and aniline hydroxylation suggests a differentiation of function, then it lends general validity to the assumption that three types of organization exist in non-induced, mixed function oxidases. It indicates the possibility therefore, of a constitutive differentiation of certain of the system's elements, a phenomenon that may be related to protein synthesis. It suggests the possibility that certain of the phenomena of induction are related to the increase of previously existing forms. While unit release and the concept of compartmentalization do not necessarily mean differentiation of all enzymatic components it would suggest the existence of several cytochrome P-450 forms and the possibility of specificity by location of other elements (20, 25). Aside from the unimodal loss in aniline and naphthalene activity, and the minimal denaturation at all levels of solubilization, the idea of specificity is further supported by considering solubility. It cannot be ruled out that partial removal of sodium deoxycholate results in conformational changes to the individual components themselves, but the data from Dowex addition suggest that insolvency rather than solubility tends to favor activity, leaving the low pellet activity following 0.5% sodium deoxycholate difficult to account for on a basis other than specificity.

While by no means a proof the idea of differential solubility is clearly testable in a number of ways. The denaturation curve of substrates which follow induction patterns different from aniline or naphthalene would provide important data. So too, would the effect of induction by various substrates on the solubility curve. Further studies on the reconstituted system to determine the maximal activity of the 0.5% sodium deoxycholate fraction in terms of its cytochrome P-450 contents would be of interest. The data clearly indicate a decrease in the specific activity from that found in unfraccionated microsomes.

With regard to reconstitution of this system (Table I) the results reported by several laboratories (1-13, 34, 35) are partially supported. The low level of cytochrome b₅ in the 0.5% fraction, raises some doubts as to its postulated role as an electron donor to the oxygenated form of cytochrome P-450, a required step in the monooxygenation reaction (36). This system is separable and capable of hydroxylating naphthalene.

The similarity in aniline and naphthalene activity loss prior to separation is, however, in disagreement. It suggests that a system functional for both substrates, can without further modification, be separated with respect to NADPH-dependent cytochrome c reductase and cytochrome P-450 fractions, indicating that reconstitution and hydroxylation are possible for both.

A differential loss in activity could possibly come about either as a result of changes in conformation inherent in separation itself or as differential denaturation through chemical treatment. The earlier data (1-11) on the sedimentation characteristics of cytochrome P-450, as well as the use of acetone extractions of the reductase fraction (1) suggest extensive solubilization of the latter, not the former. While both components in these studies are by centrifugal definition soluble, they are nonetheless high aggregates as revealed by gel filtration and analytical centrifugation (34), suggesting that the major difference lies in the reductase fraction.

Whether the above, coupled with the ability of Dowex to increase the levels of cytochrome P-450 and apparently to enhance hydroxylation as well as reductase activities (Table I), indicate a previously unsuspected specificity lost on extensive solubilization remains an interesting question.

Panfili et al. (37) have demonstrated that the efficiency of reduction of cytochrome b₅ by NADPH-dependent cytochrome b₅ reductase is related to aggregate size and a possible functional role for such aggregation has been indicated (38). Furthermore, it has been shown that it is possible to prepare a NADPH-dependent cytochrome c reductase which is inactive against cytochrome P-450 (30).

The function of phospholipid is of interest in this regard. Phospholipid was not added to these preparations and must be presumed sufficiently present to obtain the levels of activity demonstrated. It is also possible that the small amounts of sodium deoxycholate that remain bound to the enzyme preparations may replace the role of phospholipids by providing the proper configuration (13). The effect of additional phospholipid, its function in relation to spectral change or to aggregation of components, and its contrast in effect with Dowex have not been measured but certainly its possible association with reaggregation and activity are of interest. In this respect, it has recently been claimed (34) that although phospholipids may function in catalysis, their effect on hydroxylation is exerted without causing the formation of aggregates.

Should the extent of solubilization prove critical to activity, it can be noted that large aggregates may be purified from one another, as has already been done for cytochrome b₅ (38) and NADPH-dependent cytochrome b₅ reductase (37). Thus, an important experimental approach may be not in overextensive solubilization but in separation of high, but essentially pure, aggregates. The latter approach is now being undertaken in other laboratories (34).

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