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# THE PREPARATION OF MICROSOMAL ELECTRON-TRANSFER COMPLEXES

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SUMMARY

A membrane fraction has been prepared by incubation of microsomes for 10 min at room temperature in a solution which is 0.23% with respect to KCl and 10% with respect to *tert.*-amyl alcohol. This fraction which sediments as an infranatant layer contains electron-transfer components and activities at a purity about 2-fold greater than in the microsomes. This fraction is enriched in lipid and has the appearance of a vesicular membrane with a uniform repeating subunit structure with a diameter of about 50 Å, similar to that of the original microsome. It is suggested that the microsomal electron-transfer membrane is separable from microsomes because of its greater ability to bind lipid in the presence of *tert.*-amyl alcohol.

A protein complex containing the carbon monoxide-binding pigment (P-450) has been isolated from beef-liver microsomes. The P-450 compound is concentrated 4–5-fold in the isolated complex and the spectrum of the compound is undegraded. The complex contains the following prosthetic groups, flavin, a cytochrome of the *b* type and non-heme iron. Spectral studies indicate that CO does not react with the cytochrome *b*, but combines with another component which absorbs strongly at wavelengths shorter than 420 mμ.

A protein–lipid complex containing NADH–cytochrome *c* oxidoreductase activity has been isolated from beef-liver microsomes. The preparation of the enzyme by the successive use of *tert.*-amyl alcohol, cholate, and ammonium sulfate is described. The enzyme, purified 10- to 15-fold over the microsomes is soluble in the presence of bile acid and contains cytochrome $b_5$ and flavin (FAD) in a 1:1 ratio, phospholipid and non-heme iron.

## INTRODUCTION

Microsomal electron transfer is currently a field of intensive research. Although the microsomal electron-transfer enzymes are membrane-associated, many of them have been prepared in highly purified soluble form[1–5]. The concept of membrane-bound enzymes as complexes[6] implies that the individual protein components, for example those of the electron-transfer system of microsomes, are organized so that several

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proteins function together as a unit of integrated enzymic activity. The isolation of several distinct functional complexes has been of great value in understanding the electron-transfer chain of mitochondria[6–11]. A systematic study of the enzyme complexes of the microsomal electron-transfer system, however, has not yet been made.

We have found that with *tert.*-amyl alcohol it is possible to separate from microsomal preparations a membrane fraction containing the major electron-transfer systems of microsomes. This fraction contains the electron-transport enzymes in 2- to 2.5-fold greater purity than the microsomes and appears membranous when examined by electron microscopy. Using the microsomal electron-transfer membrane as a source material we have developed a simple method of preparation of a NADH–cytochrome *c* oxidoreductase (EC 1.6.1.1) complex from microsomes. The preparation and properties of this enzyme complex will be described in this paper.

A second major electron-transfer component of microsomes is a CO-binding pigment. That such a pigment existed was implicit in the studies of RYAN AND ENGEL[12] and was subsequently demonstrated in rat-liver microsomes by GARFINKEL[13] and KLINGENBERG[14]. This pigment, which has an absorption maximum at 450 mμ when combined with CO, has been designated "P-450" by OMURA AND SATO[15]. P-450 has been found in a variety of microsomal preparations[15–17] and even in mitochondria[18,20]. A role for P-450 in oxygenase reactions was postulated by ESTABROOK *et al.*[20] and by OMURA *et al.*[21] who found P-450 to be an essential component of the hydroxylation system of the adrenal cortex. CO inhibited these hydroxylation reactions and light reversed the inhibition. The action spectrum of the reversal by light corresponded to the spectrum of the CO complex of the pigment.

The isolation and characterization of P-450 have proved difficult. OMURA AND SATO[22] purified a pigment from rabbit liver which satisfied certain criteria of being the CO-binding pigment. The CO adduct of the pigment had an absorption maximum at 420 mμ and was therefore labelled P-420. These spectral anomalies were explained in terms of secondary changes in the P-450 after its detachment from the membrane. Spectral studies of the degraded form of the pigment have strengthened the view that P-450 is a cytochrome of the *b* type. In our preparation we have found that P-450 can be isolated and purified some 4–5-fold without degradation to P-420. Spectral studies, also described in this communication, indicate that P-450 is not a characteristic cytochrome.

## MATERIAL AND METHODS

### *Enzyme assays*

NADH–cytochrome *c* oxidoreductase and NADPH-cytochrome *c* oxidoreductase (EC 1.6.2.3) activities were measured under conditions described by GREEN AND MACKLER[23] except that the temperature was 30°. NADH, NADPH and ascorbic acid oxidase activities were measured polarographically at 30° with the Gilson Medical Electronics oxygraph. The reaction mixture contained 500 μmoles sucrose, 50 μmoles glucose, 40 μmoles phosphate buffer (pH 7.5), 20 μmoles $MgCl_2$, 5 μmoles substrate and 2–5 mg of protein in a final volume of 2.0 ml. Coenzyme $Q_2$ was added, where indicated, at a concentration of 50 μg/ml. $FeSO_4$ was added at a concentration of 5 μM to the reaction mixture in the assay of ascorbic acid oxidase. The hydroxylation of acetanilide by NADPH and molecular oxygen was assayed by the method de-

scribed by KRISCH AND STAUDINGER[24]. D-Glucose-6-phosphate phosphohydrolase (EC 3.1.3.9) activity was assayed by the method of SWANSON[25].

*Analytical methods*

Total iron was determined, after wet digestion of the sample, by the method of SMITH *et al.*[26]. Non-heme iron was estimated as the difference between total iron and heme iron. Copper was determined by the method of WHARTON AND TZAGOLOFF[27], and phosphorus by the method of CHEN *et al.*[28]. For the determination of lipid phosphorus, quantitative lipid extracts were made by the method of FOLCH *et al.*[29] and phosphorus determinations were performed on the extract. Extractions of RNA were carried out as described by MITTERMAYER *et al.*[30]. Particles were extracted two times with a mixture of 48 % acetone and 5 % trichloroacetic acid and two more times with 0.25 M perchloric acid at room temperature; the extracts were discarded. The particles were then extracted twice for 15 min with 0.5 M perchloric acid at 70°. The hot perchloric acid extracts were analyzed for RNA by the method of MEJBAUM[31]. Flavin was extracted with 4 % trichloroacetic acid from particles digested by trypsin, as described by BLAIR *et al.*[32]. Total flavin was estimated by the decrease in absorbance at 450 mμ following the addition of dithionite. A coefficient of 10.3 $mM^{-1}$ $cm^{-1}$ was used in the calculation.

FAD and FMN were separated by paper chromatography in a solvent system composed of butanol–acetic acid–water (12:3:5, v/v). The fluorescent spots were eluted from the paper; their contents were estimated by fluorometric analysis; authentic FMN and FAD were used as standards.

Cytochrome $b_5$ was estimated from difference spectrum between the oxidized and NADH-reduced forms. A coefficient of 163 $mM^{-1}$ $cm^{-1}$ was used for the difference in absorbance between 424 and 409 mμ (ref. 33). Total hemin was measured after formation of the pyridine hemochromogen derivative[34]. A coefficient of 29.5 $mM^{-1}$ $cm^{-1}$ was used for the difference in absorbance between 556 and 590 mμ. P-450 was estimated from the difference spectrum determined after bubbling CO through one of two dithionite-reduced samples. A millimolar extinction coefficient of 91 $mM^{-1}$ $cm^{-1}$ for the difference in absorbance between 450 and 490 mμ was used[35]. Protein was estimated by the biuret method of GORNALL *et al.*[36].

*Electron microscopy*

A drop of the suspension to be examined, at a protein concentration of 5 mg/ml was spread on a specimen grid covered on one side with a thin parlodion film, and on the reverse side with a light coat of carbon. The excess suspension was removed with filter paper and a drop of 1 % phosphotungstic acid neutralized with KOH and freshly filtered through a 100-Å millipore filter was pipetted onto the grid. Excess phosphotungstic acid was immediately removed with filter paper. Specimens were examined at 75 kV in the Hitachi HUll-B microscope. Micrographs were taken on Kodak contrast plates at electron optical magnifications of 25000 or 50000.

*Other methods*

The separation of subunit proteins by disc-gel electrophoresis was carried out in a system described by TAKAYAMA *et al.*[37]. Samples were extracted with aqueous acetone (10 % water) to removed phospholipid and bile acids. The extracted protein

was washed several times with a large volume of water; it was then dissolved in phenol–acetic acid–water (2:1:1, w/v/v). The sample was made 2 M in urea and applied to the top of a 50-mm tube of polyacrylamide gel (7.5 % acrylamide, 35 % acetic acid, 5 M urea). The proteins were separated at room temperature with a current of 5 mA for 1 h. The separated protein bands were visualized by staining with Amido Black.

Cholic acid, obtained from the Mann Biochemical Co. was treated with charcoal, recrystallized from ethanol, dried, and brought into 20 % (w/v) solution by the addition of 6 M KOH to pH 8.0.

Electron paramagnetic resonance spectra were obtained with a Varian instrument at a temperature of −172°.

## RESULTS

### *Preparation of microsomes*

Microsomes were prepared from beef liver by the method of Dr. S. FLEISCHER (personal communication). Beef liver was obtained from the slaughterhouse and was stored at 4° until used (usually within 3 h of removal from the animal). Adipose tissue was removed and the trimmed organ was sliced into small cubes, weighed, and suspended in 3 vol. (w/v) of a solution that was 0.25 M in sucrose and 0.01 M in $K_2HPO_4$. 1 ml of 6 M KOH was added for each 500 ml of the sucrose phosphate buffer and the tissue was blended in a Handrow blender[38] for 35 sec. After blending, the pH of the suspension was between 7.0 and 7.2. The homogenate was centrifuged for 9.5 min at 1600 × *g* in an International serum centrifuge having a capacity of 13 l. The supernatant fluid from the low-speed centrifugation was passed through a Sharples centrifuge at 63000 rev./min at a flow rate of 50 ml/min. The resulting supernatant was then centrifuged in the No. 30 Spinco rotor at 79000 × *g* for 90 min. The clear red supernatant was discarded and the yellow lipid adhering to the sides of the tube was wiped off with tissue paper. The translucent red pellet was resuspended in about one-tenth of the original volume of a solution that was 0.25 M in sucrose and 0.01 M in Tris–chloride (pH 7.5) (sucrose–Tris) and centrifuged at 79000 × *g* for 90 min. The resulting supernatant and loosely packed infranatant were decanted and the packed red pellet was resuspended in sucrose–Tris at a protein concentration of about 50 mg/ml. This microsomal preparation was frozen and stored at −20° until used.

### *Preparation of microsomal electron-transfer membranes*

Microsomes which had been stored at least 12 h at −20°, were thawed and the protein concentration was adjusted to 27 mg/ml with sucrose–Tris. The suspension was diluted with $^1/_3$ vol. of 0.9 % KCl and *tert.*-amyl alcohol was added with stirring to a final concentration of 10 %. The mixture, in samples of about 200 ml, was stirred slowly at room temperature for 10 min. The suspension was then centrifuged in cooled tubes at 79000 × *g* for 30 min at 4°. Three distinct phases were formed—a hard-packed brown residue, a red translucent infranatant layer and a clear yellow supernatant. The red material separated as a floating oil at *tert.*-amyl alcohol concentration greater than 10 % or if the centrifugation was not performed at low temperatures. Conversely, at lower alcohol concentrations, an effective separation was not

obtained. The supernatant containing neutral lipid but very little protein, was decanted and the red infranatant layer was collected by rinsing the pellet two or three times with a few ml of 0.9% KCl. The red fractions were pooled, homogenized in a teflon–glass homogenizer, and diluted to the original volume with 0.9% KCl. This homogenate was centrifuged at 79000 × *g* for 30 min; the supernatant was discarded and the loosely packed residue was suspended in sucrose–Tris. This preparation will be referred to as the microsomal electron-transfer membrane.

*Preparation of P-450*

Microsomal electron-transfer membranes split from microsomes were washed twice in 0.9% KCl and resuspended at 10 mg protein per ml in 0.25 M sucrose, 0.01 M Tris–chloride (pH 7.5) (sucrose–Tris). A solution of potassium cholate (20%, w/v, in cholic acid) was added to give a final concentration of 2.0 mg cholic acid per mg of protein. The clarified solution was centrifuged for 10 min at 78000 × *g*. This centrifugation caused a separation of a small amount of floating yellow material (lipid) which was removed with tissue paper. The supernatant fluid was further fractionated with a solution of ammonium sulfate saturated at 4° and neutralized to pH 7.0. Ammonium sulfate was added to 40% saturation and the precipitated protein was recovered after centrifugation at 78000 × *g* for 10 min; it was then dissolved in sucrose–Tris. Two additional ammonium sulfate fractions were obtained at 50% and 73% saturation, respectively. The supernatant fluid after 50% saturation contained little spectrally detectable P-450. The precipitate obtained at 50% saturation contained most of the P-450 pigment. This material was soluble in sucrose–Tris. The material that separated at 73% saturation appeared as a floating layer and was used as a source of NADH–cytochrome *c* oxidoreductase.

TABLE I

PURIFICATION OF THE P-450 COMPLEX FROM BEEF-LIVER MICROSOMES

| *Fraction* | *Protein* | | *P-450* | | *P-450* (*mμmoles/mg protein*) |
|---|---|---|---|---|---|
| | *Total mg* | % | *Total mμmoles* | % | |
| Microsomes | 7300 | 100 | 2480 | 100 | 0.34 |
| Microsomal electron-transfer membranes | 1150 | 15.8 | 940 | 38 | 0.82 |
| 0–40% ammonium sulphate | 210 | 2.9 | 244 | 9.9 | 1.17 |
| 40–50% ammonium sulphate | 174 | 2.4 | 302 | 12.2 | 1.75 |
| 50–70% ammonium sulphate | 166 | 2.3 | 23 | 0.9 | 0.14 |

Table I summarizes data on the purification and recovery of P-450 from beef-liver microsomes. In later fractionations the method was modified, cuts being made at 45% and at 50% saturation with respect to ammonium sulfate. P-450 was concentrated to about the same extent in each fraction. The specific concentration of P-450 in these fractions ranged from 1.4 to 1.5 mμmoles per mg protein, which indicated a 4–5-fold purification of the pigment. Refractionation of the highest purity material with cholate and ammonium sulfate did not enable us to obtain a purer preparation; rather it invariably lead to some conversion of P-450 to P-420.

*Preparation of NADH–cytochrome c oxidoreductase*

After precipitation of the remaining protein with 73 % saturated ammonium sulfate the infranatant solution was syringed off and the red viscous material was suspended in sucrose–Tris at a protein concentration of about 10 mg/ml. The turbid suspension was centrifuged at 105000 × *g* to remove a brown hard-packed pellet containing most of the residual P-450. The optically clear red supernatant fluid, designated $R_4$, was dialyzed against 100 vol. of 0.05 M Tris–HCl buffer (pH 8.0) for 3 h to remove residual salt. The dialyzed solution was then refractionated with ammonium sulfate. Saturated ammonium sulfate solution (at 4°) was added to 50 % saturation and the suspension was centrifuged at 105000 × *g* for 10 min. The brown residue was discarded. The ammonium sulfate concentration was then raised to 58 % saturation the NADH–cytochrome *c* oxidoreductase complex, designated $R_2B$, being precipitated. This protein fraction was orange in color and was soluble in sucrose–Tris. The supernatant fluid from $R_2B$ was red in color and was rich in cytochrome $b_5$, cholate, and phospholipid.

A typical fractionation is documented in Table II. The data show the distribution of total protein and of NADH–cytochrome *c* oxidoreductase activity among the fractions described above. Per mg of protein the NADH–cytochrome *c* oxidoreductase activity in the microsomal electron-transfer membranes was concentrated 2-fold above its concentration in the microsomes. In fraction $R_4$, obtained by dissolving microsomal electron-transfer membranes in cholate and fractionating with ammonium sulfate between 50 and 73 % saturation, the concentration of the enzyme

TABLE II

PURIFICATION OF NADH–CYTOCHROME *c* OXIDOREDUCTASE

Total activity is expressed as the number of μmoles of cytochrome *c* reduced per min. Specific activity is expressed as the number of μmoles of cytochrome *c* reduced per min per mg protein. $R_4$ is a fraction obtained from microsomal electron-transfer membranes. The membrane was dissolved in cholate (2 mg potassium cholate per mg protein) and $R_4$ was precipitated between 50 and 73 % saturation with ammonium sulfate; $R_2B$ was obtained from $R_4$ by fractionation with ammonium sulfate. It precipitated between 50 and 58 % saturation with ammonium sulfate.

| *Fraction* | *Total protein (mg)* | *Total activity* | *Specific activity* |
|---|---|---|---|
| Microsomes | 7300 | 5700 | 0.78 |
| Microsomal electron-transfer membranes | 1150 | 1730 | 1.50 |
| $R_4$ | 166 | 1570 | 9.50 |
| $R_2B$ | 23 | 276 | 12.0 |

was 12-fold greater than in the microsomes. At this stage, the enzyme preparation contained lipids and cholate in relatively high amounts and was rich in cytochrome $b_5$. When the enzyme complex was precipitated between 50 and 58 % saturation with ammonium sulfate, cytochrome $b_5$, cholate, and lipid remained in the supernatant fluid. The enzymic activity was concentrated about 15-fold at this stage. Recovery of the enzyme in this final fractionation was not quantitative, however. We have been unable to obtain high enzymic activities by precipitating the complex between 50 and 58 % saturation with ammonium sulfate during the first fractionation.

*Enzymic activities of microsomes and microsomal electron-transfer membranes*

Table III summarizes some of the enzymic activities of the microsomes and the microsomal electron-transfer membrane. The rates of oxidation of NADH or NADPH by cytochrome *c*, or by oxygen, in the presence or absence of a quinone such as coenzyme $Q_2$, are all from 1.6 to 2.4 times greater in microsomal electron-transfer membranes than in microsomes. Ascorbic acid oxidase which was found associated with the microsomal fraction, was also concentrated some 2.4-fold in the microsomal electron-transfer membrane fraction. The specific activity of the hydroxylating enzyme as tested with acetanilide was reduced in the *tert.*-amyl alcohol fraction. Glucose-6-phosphatase activity was apparently destroyed by treatment with the *tert.*-amyl alcohol.

*Enzymic properties of P-450*

The enzymic activities of P-450 are also presented in Table III. NADH- and NADPH–cytochrome *c* oxidoreductase which characteristically precipitate above 50% saturation with ammonium sulfate, were present in very low amounts. The preparation had NADPH oxidase activity which was stimulated by menadione or coenzyme Q. The enzyme, however, did not catalyze the oxidation by oxygen of either reduced menadione or coenzyme Q. This activity is probably the same as the naphthoquinone-dependent NADPH oxidase purified by NISHIBAYASHI *et al.*[39]. Hydroxylation activity was tested with acetanilide as substrate. None of the fractions obtained from microsomal electron-transfer membranes catalyzed the hydroxylation of this substrate.

*Activities associated with the NADH–cytochrome c oxidoreductase*

A summary of activities found in the $R_2B$ preparation is also presented in Table III. The NADH–cytochrome *c* oxidoreductase activity (in this case an average figure from several preparations) was increased 10 fold; the oxidase and dehydrogenase activities involving NADPH were increased only 2–4-fold over the microsomes. On a protein basis other specific activities were not concentrated above those of the microsomes.

The enzyme was not inhibited by the compounds that inhibit electron transfer in mitochondria, such as antimycin $A_3$, rotenone, or cyanide. Activity was not inhibited by the iron chelator, bathophenanthroline, but it was completely inhibited by *p*-chloromercurisulfonate at levels of $5 \cdot 10^{-5}$ M.

*Properties of beef-liver microsomes, microsomal electron-transfer membranes, and electron-transfer complexes*

The compositions of the microsomal preparation, the electron-transfer membrane and the electron-transfer complexes prepared with bile acid are compared in Table IV. The protein recovery in microsomal electron-transfer membranes averaged 12.5% of the total microsomal protein in 17 preparations although recoveries as high as 18% were observed. With the exception of the two metals, iron and copper, whose specific concentrations were decreased, the specific concentrations of the other electron-transfer components for which analyses were performed were increased in the electron-transfer membrane by a factor of 1.7–3.0.

TABLE III

ENZYMIC ACTIVITIES OF MICROSOMES AND MICROSOMAL ELECTRON-TRANSFER MEMBRANE

| *Activity* | *Units* | *Microsomes* | *Microsomal electron-transfer membrane* | *P-450* | *NADH–cytochrome c oxidoreductase* |
|---|---|---|---|---|---|
| NADH–cytochrome *c* oxidoreductase | mμmoles/min·mg protein | 773 | 1877 | 130–620 | 7700 |
| NADPH–cytochrome *c* oxidoreductase | mμmoles/min·mg protein | 56 | 96 | 50–80 | 190 |
| NADH oxidase | mμatoms O/min·mg protein | 7 | 17 | 3 | 8 |
| NADPH oxidase | mμatoms O/min·mg protein | 23 | 52 | 1–10 | 28 |
| NADH oxidase (+$CoQ_2$) | mμatoms O/min·mg protein | 24 | 44 | 41 | 11 |
| NADPH oxidase (+$CoQ_2$) | mμatoms O/min·mg protein | 84 | 138 | 67–82 | 271 |
| Ascorbic oxidase | mμatoms O/min·mg protein | 84 | 195 | — | |
| Hydroxylation of acetanilide | mμmoles/h·mg protein | 9.5 | 5.6 | 0.09 | 0 |
| Glucose-6-phosphatase | mμmoles/min·mg protein | 205 | 16 | 0.08 | 0 |

TABLE IV

COMPOSITION OF MICROSOMES AND MICROSOMAL ELECTRON-TRANSFER MEMBRANE

| *Component* | *Units* | *Microsomes* | *Microsomal electron-transfer membrane* | *P-450** | *NADH–cytochrome c oxidoreductase* |
|---|---|---|---|---|---|
| Protein recovery | % of microsomal protein | 100 | 12.5 | 2.3 | 0.3 |
| Total iron | mμmoles/mg protein | 11.4 | 8.0 | 4.3–6.9 | 4.3 |
| Total heme | mμmoles/mg protein | 2.0 | 3.4 | 2.0–3.2 | 0.91 |
| Copper | mμmoles/mg protein | 2.0 | 1.1 | 0.4–0.7 | 0 |
| Total flavin | mμmoles/mg protein | 0.63 | 1.23 | 0.8–1.0 | 1.05 |
| Cytochrome $b_5$ | mμmoles/mg protein | 0.46 | 1.41 | 0.05–0.6 | 0.86 |
| P-450 | mμmoles/mg protein | 0.33 | 0.75 | 1.4–1.5 | 0–0.05 |
| RNA | μg/mg protein | 42.2 | 14.3 | — | — |
| Total phosphorus | μg/mg protein | 21 | 46 | 3.9–4.6 | 11.3 |
| Lipid phosphorus | μg/mg protein | 17 | 44 | — | 10.2 |

* The analyses reported in this column were prepared on the fraction which precipitated between 45 and 50% saturation with ammonium sulfate.

Figs. 1A and 1B show the absorption spectra of microsomes and of microsomal electron-transfer membranes after solubilization with cholate. Both preparations contained cytochrome $b_5$ and another component with a cytochrome *b*-type spectrum which was not reducible by NADH. The peaks of the absorption bands in the microsomal electron-transfer membranes were sharper due to the removal of a yellow lipid material.

The fact that the RNA content per mg of protein decreased from a value of 42 μg in the microsomes to about 14 μg in the microsomal electron-transfer membranes indicates that RNA-containing material was not purified with the latter.

Lipid phosphorus was found to be concentrated in the microsomal electron-transfer membranes. Thus the specific phosphorus content increased from 17 μg/mg protein in the microsomes to 44 μg/mg protein in the membrane fraction. We can estimate the lipid content of microsomes to be about 34% by weight, and that of microsomal electron-transfer membranes to be about 57% assuming the concen-

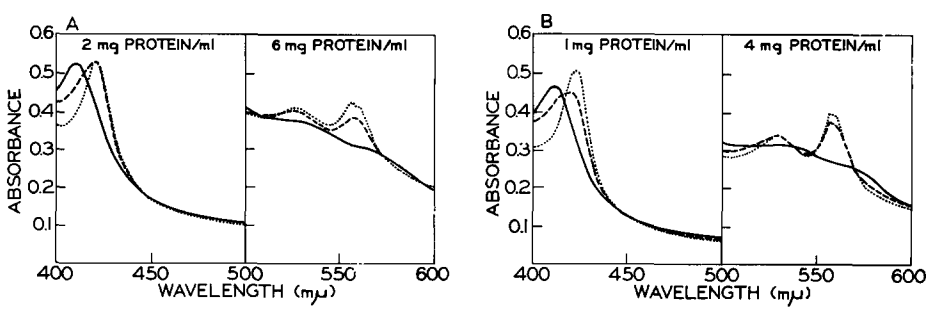



Fig. 1A. Direct spectra of microsomes. Samples were suspended in a solution 0.25 M in sucrose, 0.01 M in Tris–acetate (pH 7.5) and 1% in potassium cholate. ———, oxidized ; – – –, reduced by NADH; ·····, reduced by dithionite.

Fig. 1B. Direct spectra of microsomal electron-transfer membranes. Samples were suspended in the same sucrose–Tris solution as in Fig. 2a. ———, oxidized; – – –, reduced by NADH; ·····, reduced with dithionite.

tration of lipid phosphorus to be 33 $\mu$g per mg of lipid as calculated for mitochondrial phospholipid[40]. This increase in the lipid content gives rise to a decrease in the density of the membrane thus permitting an easy separation of microsomal electron-transfer membranes from microsomes. To determine whether or not the former is a free constituent of microsomes, both materials separately as well as a mixture of the two were subjected to density-gradient centrifugation in a continuous sucrose gradient. The results are presented diagrammatically in Fig. 2. Microsomal electron-transfer membranes formed a homogeneous band at a density of 1.08 with only a trace amount of material of higher density. Microsomes centrifuged in the same gradient showed no band at 1.08 but consisted mainly of material of a density greater than 1.16. When the two were mixed together centrifugation provided an effective separation with no apparent intermingling of the two fractions.

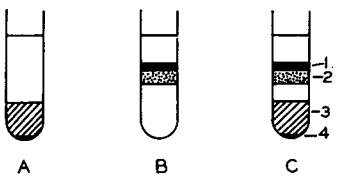



Fig. 2. Diagrammatic representation of a density-gradient centrifugation of microsomes and microsomal electron-transfer membranes. The sucrose gradient was formed with a continuous-mixing device and was continuous from 1.05 to 1.15. Centrifugation was at 30000 rev./min for 4 h in the SW 39 spinco rotor. A, microsomes; B, microsomal electron-transfer membranes; C, microsomes *plus* microsomal electron-transfer membranes. Band 1 at density 1.08 was found characteristic of microsomal electron-transfer membranes. Bands 3 and 4 were diffuse and accounted for very little protein. Band 4 was the major microsomal band.

*Ultrastructure of microsomes and microsomal electron-transfer membranes*

Fig. 3 is an electron micrograph of a typical field of the microsomal preparation. A heterogeneous population of tubular, vesicular or less well-formed structures may be discerned. Of the latter, all have a granular appearance attributable to repeating subunit particles with diameter of the order of 50 Å. The fact that these can also be seen at the edges of many of the smoother structures (inset) suggests that the 50-Å repeating unit prevails in all species of structure seen. Differences in gross appearance of these structures would, therefore, result from differences in the extent to which the repeating units were nested together.

Fig. 4 shows a typical field from the microsomal electron-transfer membrane preparation. The prevailing structure is of substantially larger diameter than the microsomes and exhibits large interior areas where structure is either absent or unstained by phosphotungstic acid. Once again, however, the size of the repeating unit at the edges is close to 50 Å, as may be verified by examination of the inset.

The enrichment in lipid in the microsomal electron-transfer membrane fraction, relative to the original microsomes, finds a corollary in the appearance of myelin figures in this fraction (Fig. 5). These also are frequently lacking in discernible interior structure. A repeating unit of about 50 Å is in evidence, though smaller units are also found.

*Composition of the P-450 complex*

A list of the components of the P-450 preparation is also presented in Table IV. The heme component of the preparation is protohemin exclusively. The spectrum

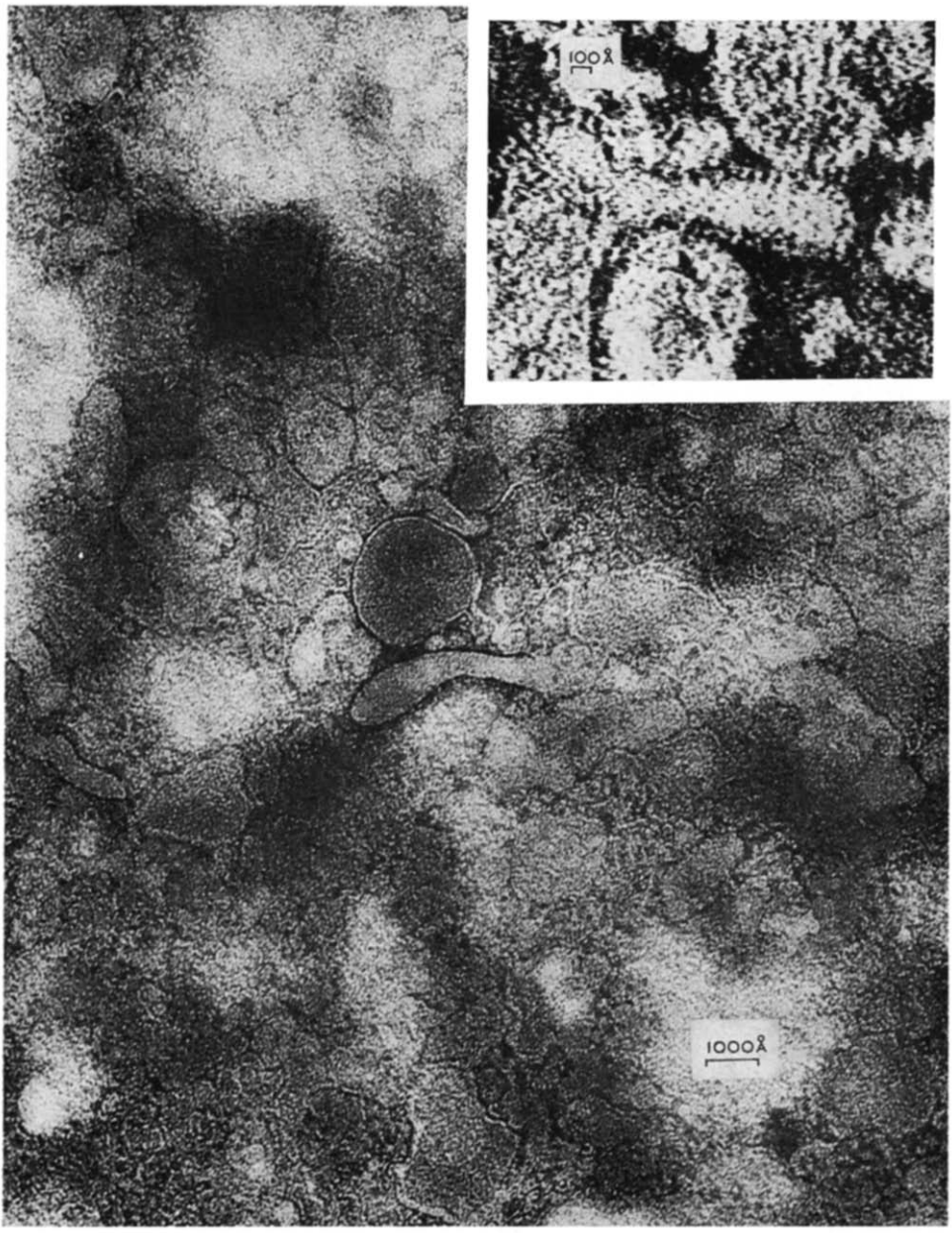



Fig. 3. Liver microsomes, negatively stained with 1% phosphotungstic acid (× 76 200); inset × 238 000.

of its pyridine hemochromogen is presented in Fig. 6. Of the total heme only a small fraction was reducible by NADH; only about half of the heme could be accounted for as P-450 when the extinction coefficient of OMURA AND SATO[22] was used. The flavin content was 0.8–1.0 mμmole per mg protein. The non-heme iron content varied between 2 and 4 mμmoles per mg protein and the concentration of non-heme iron

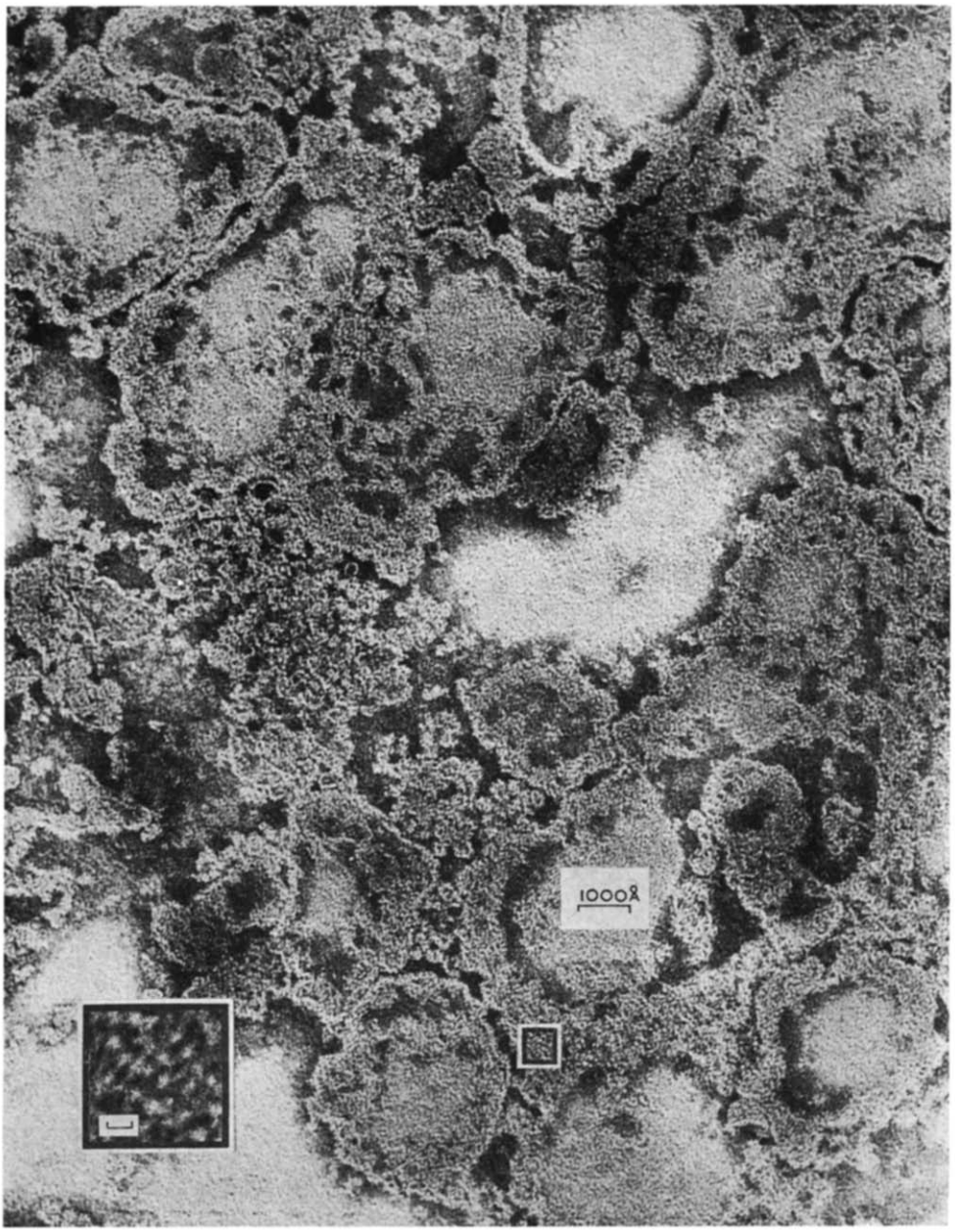



Fig. 4. Microsomal electron-transfer membrane negatively stained with 1 % phosphotungstic acid (× 77 300); inset × 386 000; inset marker 100 Å.

was generally higher in the lower ammonium sulfate fraction (45 %). Copper was present in trace amounts. The lipid phosphorus content of the preparation was 3.9–4.6 μg phosphorus per mg protein. This value corresponds to about 12 % lipid by weight if an equivalent amount of phosphorus is assumed in microsomal phos-

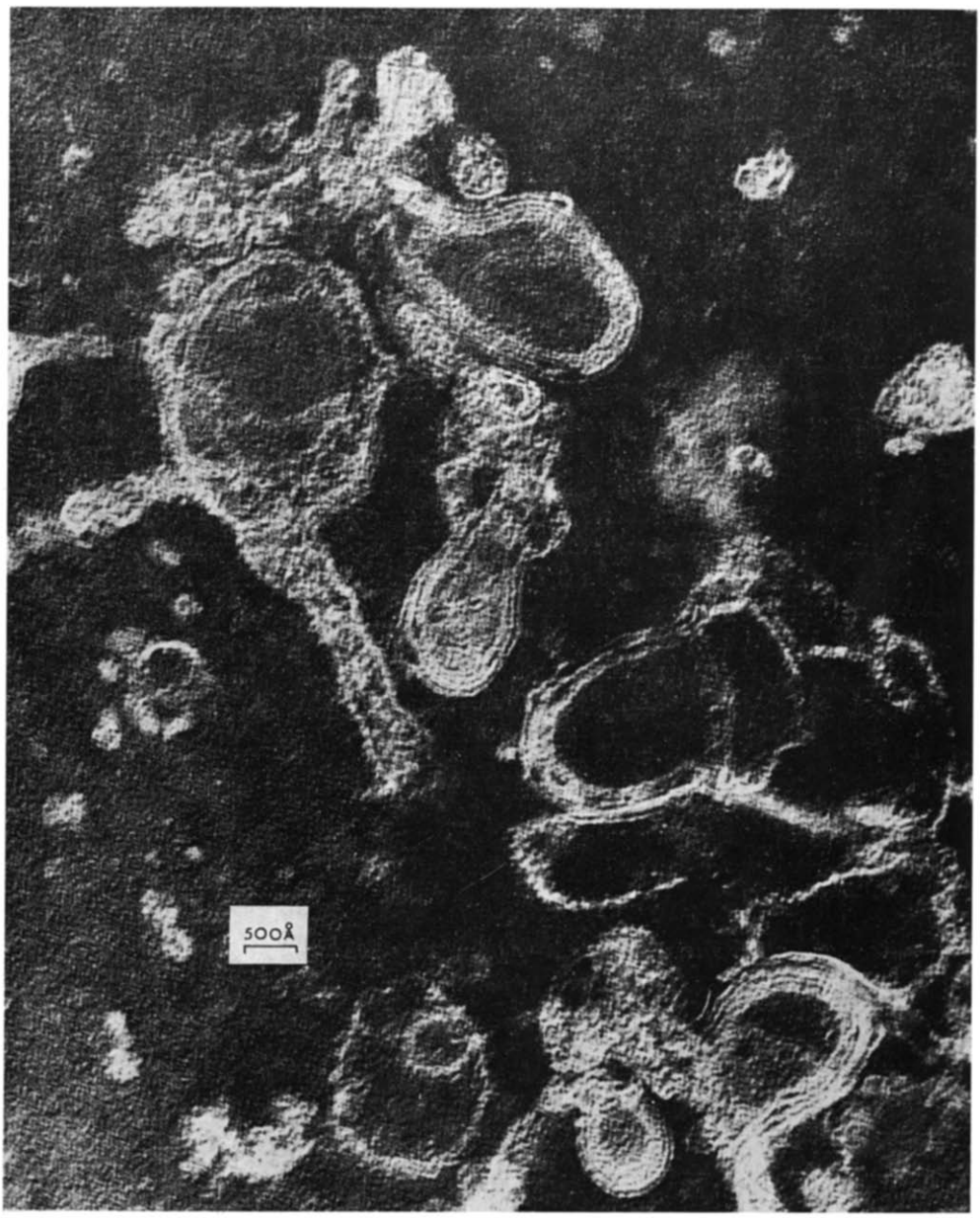



Fig. 5. Myelin figures found in microsomal electron-transfer membrane fraction (× 160000).

pholipids as was reported for mitochondrial phospholipids by FLEISCHER *et al.*[40].

The trace amounts of copper detectable in P-450 could be effectively removed by a brief dialysis (3 h) against $10^{-2}$ M KCN or $10^{-3}$ M EDTA. The fact that the amount of P-450 in the dialyzed preparation was undiminished, indicates that copper is not essential for the CO-binding property.

*Composition of the NADH–cytochrome c oxidoreductase complex*

The composition of our preparation $R_2B$ is also defined in Table IV. The concentration of cytochrome $b_5$ in the enzyme was about 1 mμmole per mg of protein and accounted for at least 94 % of the total cytochrome. P-450 was virtually absent from the preparation. Flavin was present in the complex at a level of about 1 mμmole per mg of protein and in a (approximate) 1:1 ratio with cytochrome $b_5$. At least 98 % of the flavin was in the form of FAD. Non-heme iron was present at a concentration of about 3 mμmoles per mg of protein but copper was absent. The complex contained about 11 μg of phosphorus per mg of protein, of which 10 μg were phospholipid phosphorus. On the assumption that there were 33 μg of phosphorus per mg of phospholipid (a figure determined for mitochondrial phospholipid[40]) the preparation was calculated to contain 24 % of phospholipid by weight.

Both direct and differences spectra of a typical $R_2B$ preparation, in the oxidized and dithionite-reduced forms, are presented in Figs. 7a and 7b. The major spectral

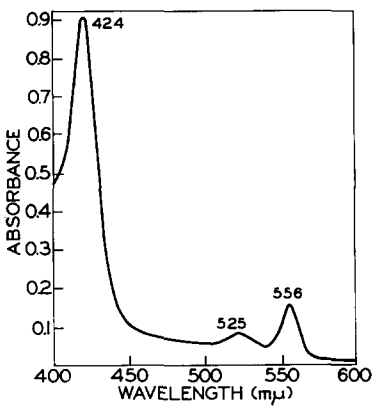



Fig. 6. Absorption spectrum of the pyridine hemochromogen of the P-450 complex. The protein was extracted first with acetone, then with acid acetone. The acid acetone extract was dried, the aqueous residue neutralized and dissolved in a 1:1 mixture of 0.2 M KOH and pyridine. The spectrum was taken following reduction by dithionite.

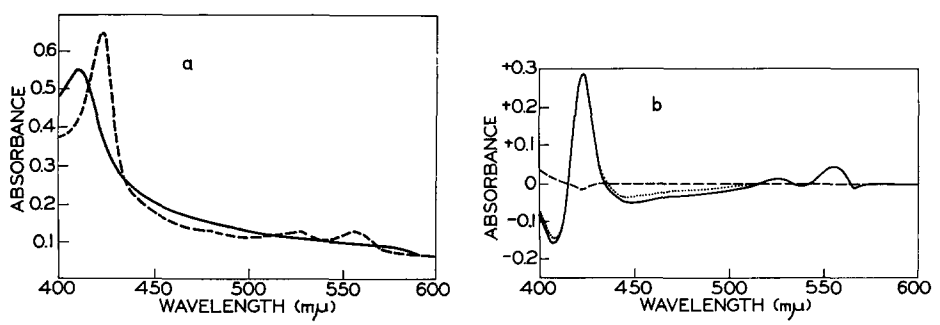



Fig. 7a. The direct absorption spectrum of fraction $R_2B$ (the NADH–cytochrome $b_5$ reductase complex): ———, chromophore in the oxidized state; – – – –, chromophore reduced with dithionite. The protein concentration was 1.6 mg/ml.

Fig. 7b. Difference spectra between the oxidized and reduced forms of $R_2B$: ———, oxidized form *versus* the dithionite-reduced form; ·····, oxidized form *versus* the NADH-reduced form; – – – –, dithionite-reduced form *versus* that treated with dithionite *plus* CO. The protein concentration was 3 mg/ml.

contribution was provided by cytochrome $b_5$ although bleaching was also evident in the region of non-heme iron absorption.

When the preparation was dissolved in phenol–acetic acid, and was subjected to electrophoresis on polyacrylamide gel, at least 10 protein bands were resolved. These data indicate that the enzyme is a complex made up of several subunit proteins; in this respect it resembles the complexes of the electron-transfer chain of mitochondria[37].

*Relationship of cytochrome $b_5$ to NADH–cytochrome c oxidoreductase.*

The essentiality of cytochrome $b_5$ for enzymic activity was evaluated from the study of an enzyme preparation in which the concentration of cytochrome $b_5$ was lower than that of flavin. This change in ratio was accomplished by repeated fractionation of the enzyme with ammonium sulfate. The concentration of flavin was thus increased from 1.1 mμmoles per mg of protein in the first fractionation to 2.3 mμmoles per mg of protein after the third fractionation.

Although the active enzyme precipitated as a pellet at 58 % saturation with ammonium sulfate, the cytochrome $b_5$ when split from the complex, precipitated as a floating oil only at a much higher concentration of ammonium sulfate. Thus, an effective separation was obtained between the particulate enzyme and free cytochrome $b_5$. The cytochrome $b_5$ forming an integral part of the complex was reduced to a level about one-half that of the flavin in two of these fractions. The enzymic activities in the cytochrome $b_5$-deficient fractions were high. They were not, however, increased concomitantly with the increase in the flavin content compared with fractions in which the ratio of cytochrome $b_5$ to flavin was greater than 1. In Fig. 8, activity is plotted against flavin content both for those fractions which had a heme to flavin ratio of 1 and for others in which the ratio was less than 1. There was greater activity per equivalent of flavin in those fractions in which the cytochrome $b_5$ to flavin ratio was greater than 1.

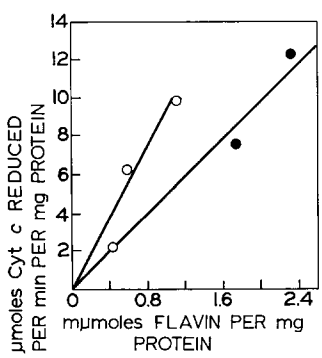



Fig. 8. Relation between activity, flavin, and heme to flavin ratios. ●, heme to flavin ratios less than 1; ○, heme to flavin ratios greater than 1.

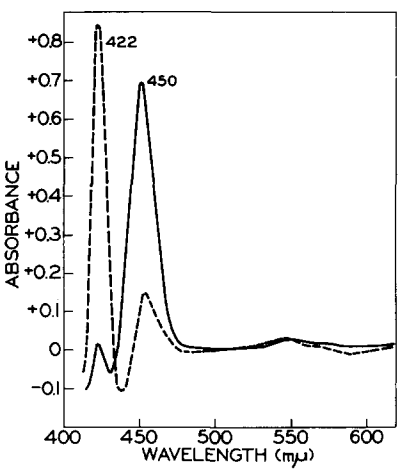
We have attempted to prepare two separate complexes from $R_4$, one containing cytochrome $b_5$ and one containing the flavin enzyme. We have not succeeded, however, in lowering the heme to flavin ratio below 0.5. We have been unable to stimulate cytochrome *c* reductase activity significantly by recombining fractions rich in cyto-

chrome $b_5$ with fractions rich in flavin. These observations lead us to believe that only one complex exists in the preparation and that it contains both cytochrome $b_5$ and the flavin enzyme.

*Spectral properties of P-450*

Fig. 9 shows the CO difference spectrum of purified P-450. The carbon monoxide adduct was formed by bubbling CO through one of two samples of P-450 which had previously been reduced with dithionite. The same spectrum was obtained when the dithionite was added after, rather than before exposure of the pigment to CO. The spectrum of the pigment was not altered during the purification, the spectrum of its CO adduct being identical in the isolated form of the pigment and in the microsomes. Fig. 9 also shows that deoxycholate at a concentration of 1 % causes a conversion of P-450 to the P-420 pigment as previously noted by OMURA AND SATO[15].

Direct absorption spectra of P-450 after reduction either by NADH, or by dithionite and treatment with CO are presented in Fig. 10. The spectrum of the oxidized pigment showed a broad Soret peak at 423 mμ. NADH reduced the small amount of



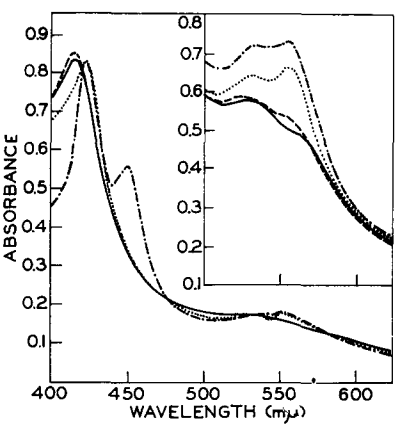




Fig. 9. Difference spectra of the CO adduct of the P-450 pigment. A preparation of P-450 was reduced with a few grains of dithionite. CO was bubbled through one-half of the solution for 5 sec. The difference spectrum is indicated by the solid line. To a parallel sample deoxycholate was added to a final concentration of 1 %. The CO difference spectrum of this sample is indicated by the broken line. The protein concentration in both samples was 5.6 mg/ml.

Fig. 10. Direct absorption spectra of the P-450 pigment. The protein concentration was 2.1 mg/ml and 11 mg/ml (in the insert); ———, oxidized; ------, reduced with NADH; ·····, reduced with $Na_2S_2O_4$; -·-·-·-, reduced with $Na_2S_2O_4$ and treated with CO.

cytochrome $b_5$ which was present in the preparation. Dithionite reduced the major cytochrome component. The α band of this cytochrome had a maximum at 556 mμ and the Soret band was maximal at 423 mμ. The spectrum of the component which was reducible by dithionite was more clearly evident in the difference spectrum after reduction by NADH and dithionite (*cf.* Fig. 11). This component had an unusual cytochrome spectrum with an α band peaking at 557 mμ, a broad Soret band at 527 mμ, and an asymmetric shoulder at 440 mμ. The calculated ratio of the absorbance

at 427 mμ to that at 557 mμ was 3.8, an exceptionally low value for a cytochrome of the *b* type.

The degradation product, P-420, which was obtained by adding deoxycholate to the preparation was partially reduced by NADH; this can be seen by the shift of the maximum of the Soret band from 414 mμ to 418, as shown in Fig. 12. The direct spectrum of the CO adduct of P-420 after reduction by dithionite was identical to that reported by OMURA AND SATO[35].

Fig. 10 shows the relationship of the 450-mμ band in the CO complex to the overall spectrum of P-450. The appearance of the band absorbing maximally at 450 mμ after treatment with CO does not cause any shift in or loss of the absorbance of the Soret band at 423 mμ. The superimposition of the spectra after reduction of the pigment by dithionite, in the Soret region between 423 and 438 mμ, both before and after treatment with CO, makes it unlikely that the cytochrome component is the reacting species. Furthermore, it is evident from the spectral changes that the component which reacted with CO had its main absorption band at shorter wavelengths (420–400 mμ), where a pronounced bleaching occurred after treatment with CO. This effect was not observed in the P-420 preparation (*cf.* Fig. 12); in this case CO intensified the Soret band at 422 mμ. The fact that the concentration of cytochrome $b_5$ in this preparation was less than 0.05 mμmole per mg protein, makes any contribution of cytochrome $b_5$ to the spectrum insignificant.

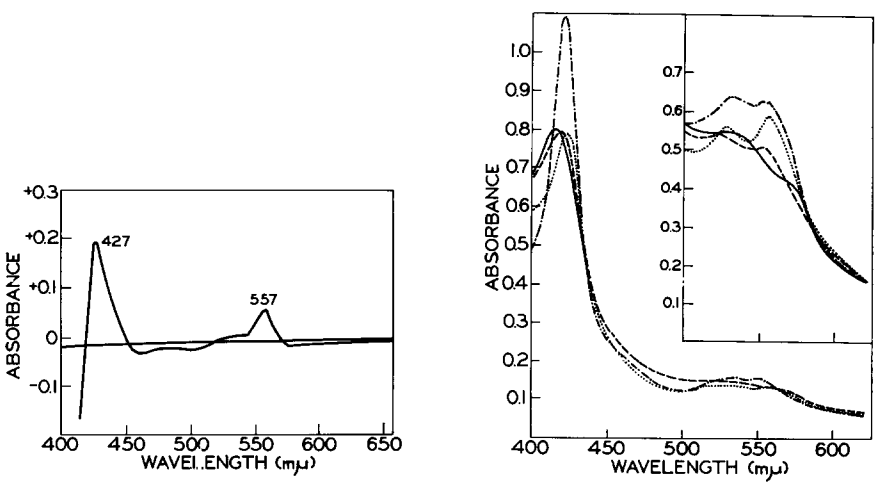



Fig. 11. Difference spectra of the P-450 complex reduced with $Na_2S_2O_4$ and with NADH. The protein concentration was 2.1 mg/ml.

Fig. 12. Direct absorption spectra of P-450 in the presence of 0.5% potassium deoxycholate. The protein concentration was 2.1 mg/ml and 11 mg/ml (in the insert). ———, oxidized; ------, reduced with DPNH; ·····, reduced with $Na_2S_2O_4$; –·–·–·–, reduced with $Na_2S_2O_4$ and treated with CO.

*Oxidation–reduction properties of P-450*

P-450 was not reduced by NADH or NADPH under aerobic conditions. The CO derivative, however, was formed slowly when the preparation was reduced with NADPH under anaerobic conditions. In this experiment (*cf.* Fig. 13), a sample of P-450 was placed in a 3-ml cuvette with a side arm and was sealed with a rubber

serum cap. Oxygen was removed by a series of evacuations and flushings with CO. A reference sample was withdrawn with a syringe. NADPH was then added from the side arm and spectra were recorded after various periods of incubation.

In agreement with the findings of OMURA AND SATO[15] P-450 was easily autoxidizable. This was determined by an experiment in which P-450 was first reduced with dithionite and then reoxidized with oxygen. When the preparation was treated with CO, no complex formation was detected spectrally. A second reduction with dithionite produced the typical band at 450 mμ.

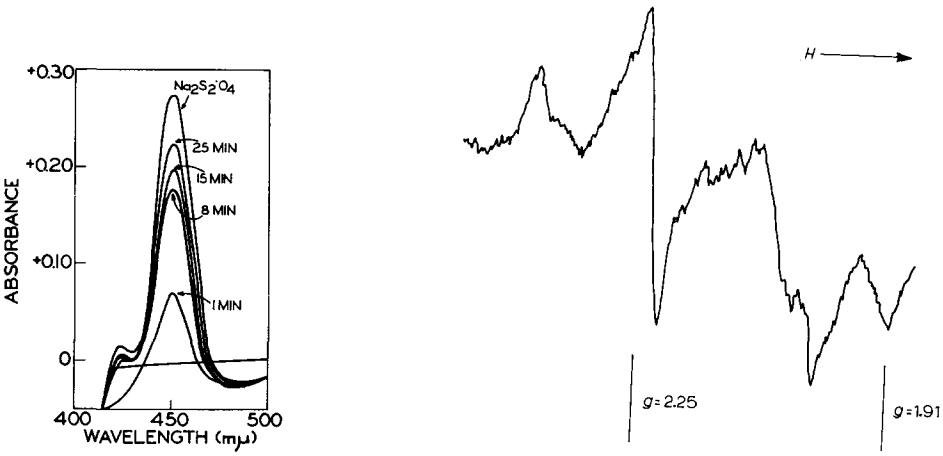



Fig. 13. Reduction of the P-450 complex with NADH. A cuvette containing a solution of P-450 (2 mg/ml) was evacuated and flushed with CO. A sample was removed from the cuvette and reoxidized with oxygen; this sample served as the reference. The difference spectrum of the two was recorded as the baseline. 5 μl of a 0.05 M solution of DPNH were added to the reference cuvette and to the cuvette containing the sample under CO. Difference spectra were recorded after the indicated period of incubation at room temperature. Finally, the solution in the anaerobic cuvette was reduced with a few grains of dithionite.

Fig. 14. Electron paramagnetic resonance spectrum of the P-450 complex in the oxidized form. The sample was dissolved in a solution which was 0.01 M in Tris–acetate (pH 7.5) at a protein concentration of 27 mg/ml. The spectrum was taken with a power of 25 mW, a modulation of 6 Gauss, and a gain of 100 at −170°.

*Electron paramagnetic resonance spectrum of P-450*

The electron paramagnetic resonance spectrum of P-450 is shown in Fig. 14. The signals at $g = 2.03$ and at $g = 2.25$, described by MASON[41,42] for microsomal $Fe_x$, were present in the preparation.

*Subunit proteins of P-450*

To determine the number of protein species present in the P-450 preparation, we employed a technique recently developed by TAKAYAMA *et al.*[37] for disc-gel electrophoresis of membrane protein. A sample of P-450 was extracted in a mixture of acetone–water (9:1, v/v) to effect a removal of phospholipid and bile acid. The extracted protein was thoroughly washed several times with water and the washed protein was then dissolved in phenol–acetic acid–water (2:1:1, w/v/v). The con-

ditions for the electrophoresis are described in the legend for Fig. 15. The high resolving power of the electrophoretic technique indicated at least eight distinguishable protein bands.

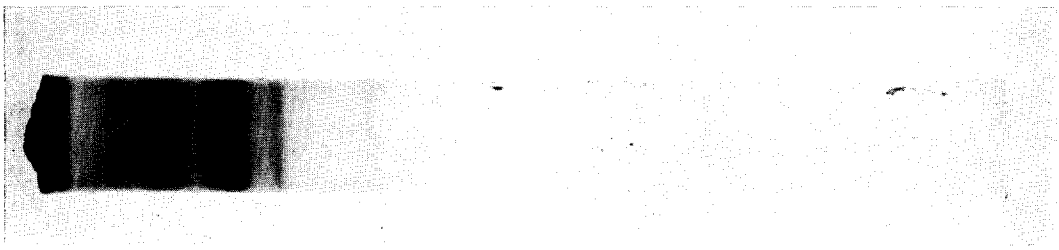


Fig. 15. Pattern of the proteins of P-450 complex separated by electrophoresis on polyacrylamide gel. The protein was extracted with 90% acetone and dissolved in phenol–acetic acid–water (2:1:1, w/v/v) in 2 M urea. A sample was applied on a polyacrylamide gel 5 mm in height. The gel consisted of 7.5% acrylamide, 35% acetic acid and 5 M urea. The proteins were separated at room temperature for 1 h with a current of 5 mA per tube and stained with Amido Black.

## DISCUSSION

Membrane-bound enzymes may be fractionated by several means. One approach involves the extraction of the protein into water after removal, or cleavage, of the phospholipid associated with the membrane[1–5,43,44]. Artificial activities are often induced in such enzyme preparations whereas the activities exhibited *in vivo* may be lost[4,43,44]. An alternative approach to this problem is by way of isolating the enzyme in a larger unit containing phospholipid[8–11]. This alternative tactic has proved to be particularly successful in the dissection of the mitochondrial electron-transfer chain[6–11].

The electron-transfer enzymes of microsomes have been fractionated following lipid extraction[1–5]. Cytochrome $b_5$ reductase and cytochrome $b_5$ are two such enzymes which have been purified after exposure of the particles to the action of phospholipase. Interaction between these two enzymes, resulting in transfer of electrons from NADH to cytochrome *c*, has led to the idea that this sequence functions *in vivo*[45].

This communication described an effort to apply the alternative procedure to the isolation of microsomal enzymes. It has been our experience that fractionation with salt in the presence of bile acids or detergent does not provide sharp separation of microsomal enzymes; and moreover, spectral studies are complicated by the presence of a yellow lipoidal material found in all fractions. Treatment of microsomes with *tert.*-amyl alcohol, therefore, has three advantages: (1) it provides an initial 2-fold concentration of electron-transfer activities; (2) it removes the lipoidal material which causes spectral interference; (3) it provides, in sufficient yield, a structural unit in which the electron-transfer complexes are amenable to further purification by fractionation with bile acids, and salt.

The electron-transfer components and activities are purified in the electron-transport membrane by a factor averaging about 2; on the basis of this purification, microsomal electron-transfer membrane could account for at most 50% of the starting protein. One possibility is that a particular species of microsome is separated from other species in the presence of *tert.*-amyl alcohol. However, it is also possible that microsomal electron-transfer membrane represents a membrane fraction split from

a more complex microsomal membrane. We find this alternative more plausible. A similar type of fractionation has been achieved by ERNSTER *et al.*[46] using deoxycholate as the disrupting agent.

Microsomal electron-transfer membrane has a significantly higher lipid content (57 %) than do the whole microsomes (34 %). Since the less dense microsomal electron-transfer membrane cannot be found in microsomal preparations when subjected to density-gradient centrifugation, a redistribution of lipid must have occurred during exposure of the microsomes to *tert.*-amyl alcohol. FLEISCHER AND BRIERLEY[47] have shown that free phospholipid equilibrates with membrane-bound phospholipids in the presence of *tert.*-amyl alcohol. We suggest that it is the greater ability of microsomal electron-transfer membrane to bind phospholipid that permits its separation from other proteins as an infranatant layer of reduced density.

The isolation of an electron-transfer membrane from beef-liver microsomes has made it feasible to purify a P-450 complex by fractionation with cholate and ammonium sulfate. The procedure is simple and yields a final preparation which contains little of the P-420 degradation product.

Analysis of the complex for electron-transfer components has shown the presence of flavin, protohemin, non-heme iron, and trace amounts of copper. The copper, however, could be removed with chelators such as cyanide or EDTA and the copper-free preparation was unchanged in its ability to react with CO. The presence of oxidation–reduction components other than P-450 makes us believe that this preparation is comparable to the enzyme complexes isolated from the electron-transfer chain of mitochondria[6–11]. In this sense it is difficult to compare the P-450 reported here with the P-420 isolated by OMURA AND SATO[22] which may be a split product of a larger enzyme unit. Our own attempts to obtain a more highly purified P-450 complex have not been successful. Thus a second refractionation was accompanied by spectral denaturation without substantial increase in the purity of the pigment.

The major heme component of the P-450 complex is a cytochrome characterized by an atypical spectrum. Since the heme prosthetic group could be extracted with acid acetone, and its pyridine hemochromogen was spectrally identical to that of protohemin, the cytochrome component is probably of the *b* type. It differs from most cytochromes of this group[48–50] in having unusually broad and weakly absorbing, $\alpha$ and Soret bands.

OMURA AND SATO[22] identified the cytochrome of their P-420 preparation as the CO-binding pigment. The main cytochrome component of the P-450 complex described here is probably the same as the P-420 cytochrome isolated by OMURA AND SATO since the spectrum of our preparation in the presence of deoxycholate is identical to theirs. Our spectral data, however, do not support the conclusion that CO reacts with the cytochrome in the P-450 complex. Thus, while there is a rapid reduction of the cytochrome with dithionite, subsequent exposure to CO has no apparent effect on the absorption in the $\alpha$ and Soret bands, even though a new band with a maximum at 450 m$\mu$ is evident. More striking, however, is the strong bleaching effect observed in the CO complex below 420 m$\mu$. This observation suggests the presence of another chromophore which reacts with CO and has its greatest absorption in the near-ultraviolet region. Whether this component is a non-heme iron or some other, as yet unidentified, group cannot be concluded at present.

The electron paramagnetic resonance spectrum of P-450 at $-172^\circ$ revealed

the presence of the $Fe_x$ signals at $g = 2.03$ and at $g = 2.25$ (refs. 41, 42). Both signals disappeared on reduction with dithionite; reducibility by other substrates was not tested. MASON[41,42] has presented evidence indicating the separate nature of $Fe_x$ and P-450; however, the nature of $Fe_x$ has yet to be defined.

TAKAYAMA *et al.*[37] have recently devised a method of studying the subunit of particulate enzymes. We employed this method to examine the number of proteins present in the P-450 complex. We found that the electron-transfer complexes of microsomes such as P-450 and NADH–cytochrome *c* oxidoreductase are composed of multiple protein subunits. This observation is also true of the four complexes of the mitochondrial electron-transfer chain. This fact points to a basic similarity in the organization of the electron-transfer enzymes of the two membrane systems.

The abundance of P-450 in beef-liver microsomes, as well as in microsomes prepared from other sources suggests an important enzymic function for this electron-transfer complex. The CO-binding pigment of adrenal cortex has been implicated by ESTABROOK *et al.*[17,20] as the oxygen-activating enzyme in the hydroxylation of steroids at C-21. The P-450 complex prepared from adrenal cortex and that isolated from beef-liver microsomes, reported here, are remarkably alike in their spectral and oxidation–reduction properties. This leads us to believe that they share a common enzymic function.

We have isolated what we believe to be a second electron-transfer complex which transfers electrons between reduced NAD and cytochrome *c*. It contains flavin, cytochrome $b_5$, non-heme iron, phospholipid, and several subunit proteins. This enzyme preparation should prove useful in answering certain questions: for example, does the system require lipid *in vivo*; is non-heme iron a functional component; and is cytochrome $b_5$ an electron acceptor? Our preliminary observations indicate that cytochrome $b_5$ is an integral part of the complex and is essential for maximal activity. The preparation described here may be compared with at least two preparations from other laboratories. The enzyme complex contains the cytochrome $b_5$ and the cytochrome $b_5$-reductase proteins isolated by STRITTMATTER AND VELICK[2]. These proteins are components of the enzyme complex and therefore are not pure proteins. Our preparation contains, in addition to cytochrome $b_5$ and cytochrome $b_5$-reductase, phospholipid, non-heme iron, and other unidentified proteins.

Our preparation is similar to that prepared by PENN AND MACKLER[52], but the composition of the preparations, is somewhat different. In our preparation the heme to flavin ratio is 1 to 1; in the preparation of PENN AND MACKLER it is 2.5 to 1. Both preparations are rich in lipid and both contain non-heme iron. The enzyme of PENN AND MACKLER, however, is insoluble and its preparation is more time-consuming.

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