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# Preparation and Properties of a Solubilized Form of Cytochrome P-450 from Chick Embryo Liver Microsomes

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SUMMARY

A procedure for the partial purification and subsequent solubilization of cytochrome P-450 from microsomal membranes of chick embryo liver is described. Successive treatment of microsomes with Protease VII and Lubrol WX yielded a solubilized preparation with about a 1.8-fold increase in the specific content of cytochrome P-450, measured as millimicromoles per milligram of protein, with no detectable amounts of cytochrome $b_5$ and minimal activities of NADPH- and NADH-cytochrome *c* reductases. The CO difference spectrum of the solubilized cytochrome P-450 showed an absence of cytochrome P-420; however, the absolute spectrum of the CO complex of the reduced preparation displayed a peak at 420 mμ. The solubilized cytochrome P-450 obtained from chick embryo livers interacted with various substrates to give type I or type II spectra similar to those observed in other species. Cytochrome P-450 in chick embryo liver was inducible by drugs, polycyclic hydrocarbons, and steroids.

## INTRODUCTION

The liver microsomal hemoprotein cytochrome P-450 has been implicated as the terminal oxidase for the metabolism of drugs (1, 2), carcinogenic hydrocarbons (1, 2), steroids (3), and fatty acids (4, 5).

The addition of various substrates to microsomal preparations results in spectral changes, suggesting an interaction between the substrate and the hemoprotein P-450. These spectral changes are classified into two types: type I, having a maximum at about 390 mμ and a minimum at about 420 mμ, and type II, having a maximum at about 420 mμ and a minimum at about 390 mμ (6–8). Recent studies have also shown the presence of at least two different forms of P-450 in liver cell microsomes: cytochrome P-450, which is preferentially induced by treatment of animals with drugs, such as phenobarbital, and cytochrome P-448, which is preferentially induced by polycyclic hydrocarbons, such as 3-methylcholanthrene (9, 10). The two forms of P-450 can be distinguished by indirect means, such as the formation of an ethyl isocyanide–hemoprotein complex (11), an *n*-octylamine–hemoprotein complex (12), or a carbon monoxide–hemoprotein complex (9, 10). It has been shown that two hemoproteins with different turnover rates occur in microsomal CO-binding particles (13, 14), and that treatment of rats with 3-methylcholanthrene preferentially induces the hemoprotein with the longer half-life (13).

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Murphy *et al.* (15), using rate-zonal centrifugation, also demonstrated that cytochrome P-448 produced by treatment with 3-methylcholanthrene was found in larger quantities in particles having an increased sedimentation rate.

In order to study the properties of these microsomal hemoproteins, several attempts have been made to isolate and purify cytochrome P-450. Microsomal cytochrome P-450 relatively free from cytochrome $b_5$ and NADPH–cytochrome *c* reductase activity has been obtained using trypsin (16, 17), steapsin (18, 19), Nagarse (a *Bacillus subtilis* proteinase) (17), and deoxycholate (20). In all preparations except those treated with Nagarse, cytochrome P-450 was partly converted to P-420, the denatured form of the hemoprotein. Solubilization and partial purification of cytochrome P-450 have been effected by treatment of microsomes with Lubrol WX followed by Sephadex G-25 and DEAE-Sephadex column chromatography (12, 21), or by treatment with deoxycholate followed by DEAE-cellulose column chromatography (4). These methods did not increase the specific content of the solubilized P-450, in terms of millimicromoles per milligram of protein. In the latter procedure (4), moreover, 30% of the P-450 was changed to P-420. MacLennan *et al.* (22) have succeeded in obtaining a "P-450 particle" with an apparent 4–5-fold purification by treatment of microsomes with *tert*-amyl alcohol and potassium cholate.

A variety of 5$\beta$-steroid metabolites natural to man have been shown in previous studies from these laboratories to be potent inducers of $\delta$-aminolevulinic acid synthetase in the chick embryo liver or in liver cell culture (23, 24). This mitochondrial enzyme is rate-limiting in the heme-biosynthetic pathway. The relationship between steroid enhancement of $\delta$-aminolevulinic acid synthetase formation and of heme biosynthesis, in the form of cytochrome P-450, together with the usefulness of the chick embryo preparation for investigations of the microsomal drug-metabolizing enzyme system, prompted this study of the isolation and characterization of chick embryo liver cytochrome P-450.

In this study, another *B. subtilis* proteinase preparation (Protease VII) was used for the removal of cytochrome $b_5$ and NADPH–cytochrome *c* reductase from microsomal membranes. Since both Protease VII and Nagarse are crude proteinase preparations obtained from *B. subtilis* and are prepared commercially by two different laboratories—Sigma Chemical Company, St. Louis, and Nagase Company, Japan—an attempt was made to compare the abilities of these two preparations to solubilize cytochrome $b_5$ and NADPH–cytochrome *c* reductase. Lubrol WX was next employed to solubilize the membrane-bound P-450. The solubilized P-450 thus obtained was essentially free of cytochrome $b_5$, cytochrome P-420, and NADPH–cytochrome *c* reductase activity. The specific content of the solubilized P-450 was almost twice that of the original microsomes. The spectral properties of the soluble P-450 from chick embryo liver microsomes were also examined, as was the interaction of the P-450 with several model drug and steroid substances.

## MATERIALS AND METHODS

*Treatment of chick embryos.* Chick embryos 17–19 days old were used in all experiments. Drugs or steroids (1.5% solutions, w/v) in a volume of 0.2 ml were injected into the yolk sac of the chick embryo through a small hole drilled into the blunt end of the egg, which then was incubated at 37° for 24 hr unless otherwise specified. The drugs used were allylisopropylacetamide dissolved in propylene glycol, sodium phenobarbital dissolved in 0.9% NaCl, and 3-methylcholanthrene dissolved in corn oil. The steroids used were 17$\alpha$-hydroxy-11-ketopregnanolone (3$\alpha$, 17$\alpha$-dihydroxy-5$\beta$-pregnane-11, 20-dione) and etiocholanolone (3$\alpha$-hydroxy-5$\beta$-androstan-17-one), both dissolved in propylene glycol. Control chick embryos were treated with an injection of 0.2 ml of the appropriate solvent.

*Preparation of microsomal fractions from chick embryo liver.* Livers of chick embryos were perfused *in situ* through the heart with a solution containing 1.15% KCl and heparin (100 USP units/ml). The livers were then homogenized in 4–6 volumes of 0.25 M sucrose containing 0.1 mM sodium ethylene-

diaminetetraacetate with five strokes of a motor-driven glass-Teflon homogenizer. The homogenate was centrifuged at 9000 × *g* for 20 min. Microsomal pellets were obtained by centrifuging the 9000 × *g* supernatant fraction at 105,000 × *g* for 60 min. The resulting pellets were washed once with 1.15% KCl–0.1 M sodium phosphate buffer containing glycerol at a concentration of 25% (v/v). Each milliliter of suspension contained microsomes equivalent to 1 g of liver, wet weight. This preparation could be stored at −20° for 1 month without any appreciable loss of cytochrome P-450 as determined spectroscopically.

*Preparation of microsomal fractions from phenobarbital- and 3-methylcholanthrene-treated rat livers.* Female Sprague-Dawley rats weighing 200–250 g were treated with phenobarbital or 3-methylcholanthrene intraperitoneally at a dose of 75 mg/kg or 20 mg/kg daily, respectively, for 5 days. After fasting for 20 hr, the rats were killed by decapitation and their livers were perfused *in situ* with 1.15% KCl–heparin solution. Liver homogenates were centrifuged at 600 × *g* for 10 min, and microsomes were prepared as described above.

*Preparation of solubilized P-450 from chick embryo liver.* Livers were removed from approximately 200 chick embryos without perfusion and rinsed thoroughly in 1.15% KCl solution. Liver microsomes were prepared as described above and subsequently treated as follows at 0–4°.

Step I: The protein concentration of liver microsomes suspended in 0.1 M sodium phosphate buffer, pH 7.0, containing 25% glycerol, was measured and adjusted to 5 mg/ml of 0.1 M sodium phosphate buffer–glycerol solution.

Step II: Protease VII (Sigma), 10 mg/ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 25% glycerol, was added to the microsomal suspension at a final concentration of 20 μg/mg of microsomal protein. The mixture was then incubated at 4° for 15 hr in an atmosphere of $N_2$. In some preliminary experiments two protease preparations from *B. subtilis*, Nagarse and Protease VII, were compared for their ability to solubilize cytochrome $b_5$ and NADPH–cytochrome *c* reductase.

Step III: The microsomal preparation was diluted 3-fold with 0.1 M sodium phosphate buffer, pH 7.0, and centrifuged at 105,000 × *g* for 60 min. The supernatant fraction was then discarded, and the pellet obtained was resuspended in 0.1 M sodium phosphate buffer–glycerol solution, pH 7.0. The suspension was again diluted 3-fold and centrifuged at 105,000 × *g* for 60 min as described above.

Step IV: The pellet from step III was resuspended in 0.05 M sodium phosphate buffer–25% glycerol solution, pH 7.0. The protein concentration of the suspension was determined, and the protein content was adjusted to 8 mg/ml of 0.05 M sodium phosphate buffer–glycerol solution. To this diluted preparation, sufficient 5% Lubrol WX (w/v) (Arnold and Hoffman Company, Providence, R. I.) in 0.05 M sodium phosphate buffer–25% glycerol solution, pH 7.0, was added to make a final concentration of Lubrol WX in the mixture of 2.0 mg/mg of protein. The mixture was stirred mechanically at 4° for 30 min.

Step V: The mixture was centrifuged at 105,000 × *g* for 50 min. Four layers were observed. The top, turbid layer was carefully pipetted off and discarded. The next, an orange-red clear phase, was collected and saved. The pellet and the interphase between the clear phase and the pellet itself were discarded.

Step VI: To the combined orange-red clear phases, an equal volume of saturated ammonium sulfate solution (60 g of ammonium sulfate dissolved in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0, after which the pH was adjusted to 7.8 with 4 M ammonium hydroxide) was added, and the mixture was then centrifuged at 78,000 × *g* for 40 min.

Step VII: Following centrifugation, the particulate material floating on top of the supernatant fraction was removed carefully with a small spatula and placed on the wall of a test tube for a few minutes to drain any ammonium sulfate solution adhering to it. The particulate material was then dissolved in a minimal volume of 0.1 M sodium phosphate buffer–25% glycerol solution. The recovery of microsomal protein in this final preparation, which was a clear solution, was about 30%. The solubilized preparation was

devoid of cytochrome P-420, as measured by the CO difference spectrum, and cytochrome $b_5$. Very small amounts of NADH- and NADPH-cytochrome *c* reductases were present in the final preparation, as described later. This final preparation could be stored at −20° for several weeks with little loss of activity. It has been termed soluble P-450, since no cytochrome P-450 was sedimented when the particulate matter was dissolved in 0.1 M sodium phosphate buffer in the presence of 5% glycerol (specific gravity of 5% glycerol in 0.1 M sodium phosphate buffer at 4° = 1.01) and centrifuged for 120 min at 105,000 × *g*.

*Measurements of absorption spectra.* The absolute and difference spectra of microsomes and of soluble P-450 were measured with a Cary recording spectrophotometer, model 15, using microcuvettes (1-cm optical path, 4-mm width) at room temperature. For measurement of the absolute spectrum of the carbon monoxide complex of P-450, CO was bubbled into the sample cuvette for 30 sec, and then a few crystals of sodium dithionite were added to the sample. For measurement of substrate binding to solubilized P-450, 0.4 ml of the sample was placed in reference and sample cuvettes and the difference spectrum was recorded. Steroids, dissolved in dimethylsulfoxide, were added in a 20-μl volume to give a final concentration of 48 μM in the sample. The final concentrations of drugs in sample cuvettes were as follows: aminopyrine and aniline, 15 mM; allylisopropylacetamide and nicotinamide, 30 mM. The ethyl isocyanide difference spectra were determined as follows: 0.4-ml aliquots of the dithionite-reduced preparation, pH 7.0, were placed in reference and sample cuvettes; ethyl isocyanide was added to the sample cuvette at a final concentration of 1.2 mM, and the difference spectrum was recorded.

*Analytical methods.* The protoheme content was measured spectrophotometrically by the difference in absorption at 418 mμ between CO–pyridine ferrohemochrome and pyridine ferrihemochrome: 2.0 ml of 0.1 M borate buffer, pH 8.9, containing 25% pyridine, were added to 0.5 ml of sample, CO was bubbled through, and then 0.01 ml of water and a small amount of sodium dithionite were added to the sample cuvette. To the reference cuvette, 0.01 ml of 10% sodium iodide solution was added. An extinction coefficient of 116 $mM^{-1}$ $cm^{-1}$ was used to calculate the protoheme content.[2] The method is especially useful when the amount of material is limited, as in the present studies. The sensitivity of the assay is approximately 6 times greater than the pyridine hemochromogen assay of Porra and Jones (25), and the values obtained by the above method are in good agreement with the values obtained by the pyridine hemochromogen assay method (25).

Total amounts of flavin were determined fluorometrically by the method of Bessey *et al.* (26) after extraction with cold 11% trichloracetic acid.

The P-450 contents were estimated by the method of Omura and Sato (27) from the CO difference spectrum of dithionite-reduced preparations, using an extinction coefficient of 91 $mM^{-1}$ $cm^{-1}$ between 450 mμ and 490 mμ (27). The P-420 contents were estimated by the method of Omura and Sato (27) from the CO difference spectrum of dithionite-reduced preparations.

For the determination of cytochrome $b_5$, the NADH reduced minus oxidized difference spectrum was measured between 424 and 409 mμ. An extinction coefficient of 185 $mM^{-1}$ $cm^{-1}$ (27) was used to quantitate cytochrome $b_5$. The final concentration of NADH in the sample cuvette was 0.5 mM.

Protein was determined by the method of Lowry *et al.* (28), using bovine serum albumin as a standard.

The activities of NADH- and NADPH-cytochrome *c* reductases were measured according to the method of Dallner *et al.* (29), with the following modifications. The reactions were started by rapidly adding 0.02 ml of 3 mM NADH (or NADPH) to a solution of 0.05 M sodium phosphate buffer, pH 7.5, containing 30 mμmoles of cytochrome *c*, 0.2 mμmole of KCN, and 0.08 ml of a microsomal or soluble P-450 preparation (30–40 μg of microsomal protein or 2–3 mg of protein of soluble P-450). The total volume of the reaction mixture was 0.6 ml. The amount of cytochrome *c* reduced per minute in the mixture was recorded as the

[2] S. Sassa and S. Granick, unpublished results.

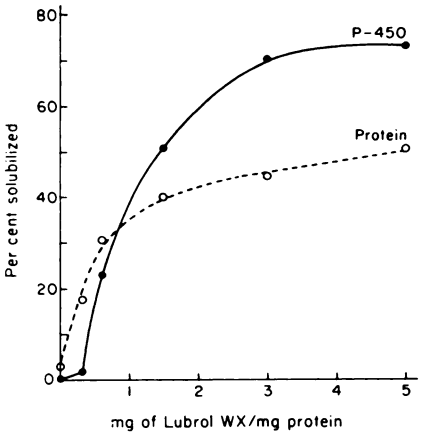


FIG. 1. *Effect of concentration of Lubrol WX on solubilization of cytochrome P-450 and protein from allylisopropylacetamide-treated chick embryo liver microsomes*

Allylisopropylacetamide was injected into the yolk sacs of 17-day-old eggs at a dose of 3 mg/embryo, followed by further incubation of the eggs for 24 hr at 37°. The protein concentration of the microsomal suspensions obtained from allylisopropylacetamide-treated chick embryo liver was adjusted to 8 mg/ml of 0.05 M sodium phosphate buffer, pH 7.0, containing 25% glycerol. After incubation with various amounts of Lubrol WX for 30 min at 4°, the mixtures were centrifuged at 105,000 × *g* for 50 min and the concentrations of P-450 and protein in the supernatant fractions were determined.

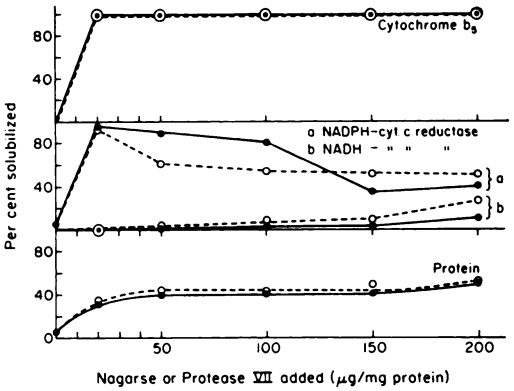


FIG. 2. *Effect of concentration of Protease VII or Nagarse on solubilization of cytochrome $b_5$, NADPH- and NADH-cytochrome c reductases, and protein from allylisopropylacetamide-treated chick embryo liver microsomes*

Liver microsomal suspensions (5.0 mg of protein per milliliter of 0.1 M sodium phosphate buffer, pH 7.0, containing 25% glycerol) prepared from allylisopropylacetamide-treated chick embryos and containing 1.4 mμmoles of P-450 and 0.438 mμmole of cytochrome $b_5$ per milligram of microsomal protein were incubated with various amounts of Protease VII (○- - -○) or Nagarse (●——●) at 4° for 15 hr under nitrogen gas. The contents of the reaction mixtures were centrifuged at 105,000 × *g* for 60 min. The percentages of cytochrome $b_5$, NADPH-cytochrome *c* reductase, NADH-cytochrome *c* reductase, and protein solubilized into the supernatant fraction were calculated from the amounts present in both supernatants and pellets.

difference in absorbance at 550 mμ between reduced and oxidized cytochrome *c*, and the amount was calculated using an extinction coefficient of 18.5 $mM^{-1}$ $cm^{-1}$ (29).

## RESULTS

*Effect of Lubrol WX on extraction of P-450 from chick embryo liver microsomes.* Figure 1 shows the effect of the concentration of Lubrol WX on solubilization of P-450 from microsomal membranes. At no concentration of Lubrol WX used in these experiments was P-420 detectable spectrophotometrically in any of the 105,000 × *g* supernatant fractions. In order to reduce the amounts of Lubrol WX in the final preparations, since this agent appears to interrupt the normal sequence of flow of reducing equivalents in microsomes (21), and to solubilize the P-450 most effectively, it was decided to use a Lubrol WX to protein ratio of 2.0 mg/mg. After treatment of chick embryo liver microsomes with Lubrol WX, 1.6–1.8-fold purification was achieved in terms of amounts of P-450 per milligram of protein.

*Effect of Protease VII on solubilization of cytochrome $b_5$ and NADPH-cytochrome c reductase from chick embryo liver microsomes.* Recently Nishibayashi and Sato (17), using Nagarse, succeeded in removing cytochrome $b_5$ and NADPH-cytochrome *c* reductase from the microsomal membranes of phenobarbital-treated rabbit livers without appreciable conversion of P-450 to P-420. In the present study Protease VII was used for this purpose, and the ability of this enzyme to solubilize cytochrome $b_5$ and NADPH-cytochrome *c* reductase was compared with

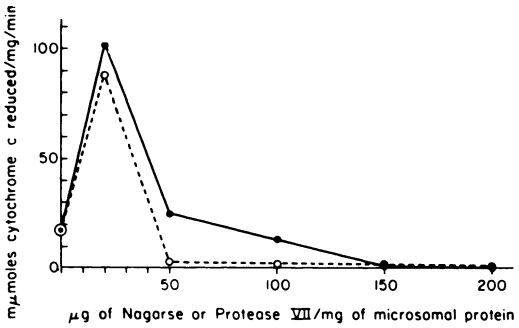


FIG. 3. *Specific activity of NADPH-cytochrome c reductase after treatment of microsomes with Protease VII or Nagarse.*

Experimental conditions were the same as described in the legend to Fig. 2. The specific activity of NADPH-cytochrome *c* reductase was calculated from the contents of NADPH-cytochrome *c* reductase and protein in the supernatant fraction and expressed as millimicromoles of cytochrome *c* reduced per milligram of microsomal protein per minute. ●——●, Nagarse-treated; ○- - -○, Protease VII-treated.

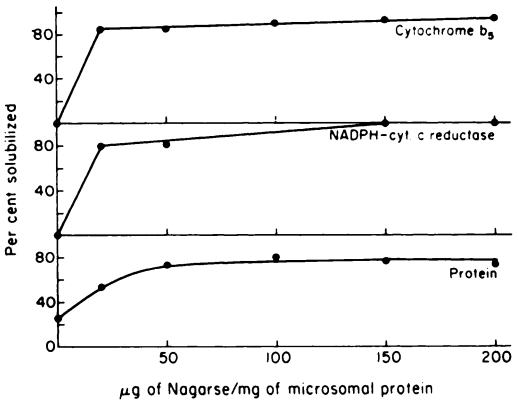


FIG. 4. *Effect of concentration of Nagarse on solubilization of cytochrome $b_5$, NADPH-cytochrome c reductase, and protein from liver microsomes of phenobarbital-treated rats.*

Rats were treated with phenobarbital intraperitoneally at a dose of 75 mg/kg/day for 5 days. Liver microsomal suspensions (5.0 mg of protein per milliliter of 0.1 M sodium phosphate buffer, pH 7.0, containing 25% glycerol) were prepared and contained 2.05 mμmoles of P-450 and 0.768 mμmole of cytochrome $b_5$ per milligram of protein. The microsomal suspensions were incubated with various amounts of Nagarse, and the percentages of cytochrome $b_5$, NADPH-cytochrome *c* reductase, and protein solubilized into the supernatant fraction were calculated as described in the legend to Fig. 2.

that of Nagarse. Anaerobic incubation of allylisopropylacetamide-treated chick embryo liver with various amounts of Protease VII was carried out in the presence of 25% glycerol at 4° for 15 hr under the conditions described by Nishibayashi and Sato (17).

As shown in Fig. 2, cytochrome $b_5$ and NADPH-cytochrome *c* reductase were almost completely released from the microsomal membranes along with 30–35% of the total microsomal protein after centrifugation of the contents of the incubation flasks at 105,000 × *g* for 50 min at concentrations of Nagarse or Protease VII as low as 20 μg/mg of microsomal protein. Since the degree of solubilization of NADPH-cytochrome *c* reductase was reduced with increases in the concentration of Nagarse or Protease VII, NADPH-cytochrome *c* reductase seemed to be partially degraded during the incubation. Further studies showed that the specific activity of NADPH-cytochrome *c* reductase, expressed as millimicromoles of reduced cytochrome *c* per milligram of protein per minute, dropped markedly with both Nagarse and Protease VII digestion at concentrations of the proteases greater than 20 μg/mg of protein (Fig. 3). However, when phenobarbital-treated rat liver microsomes were subjected to Nagarse digestion, no appreciable decrease in enzyme activity occurred following solubilization of NADPH-cytochrome *c* reductase under the same conditions used for chick embryo liver (Fig. 4). Thus it appears that the NADPH-cytochrome *c* reductase of chick embryo liver microsomes is more sensitive to protease treatment than is the reductase of rat liver microsomes. When Protease VII at a concentration of 20 μg/mg of microsomal protein was used, the solubilization of cytochrome $b_5$ and NADPH-cytochrome *c* reductase was similar to that obtained with the same concentration of Nagarse. Although cytochrome $b_5$ and NADPH-cytochrome *c* reductase appeared to be readily released from the microsomal membranes by Protease VII or Nagarse, NADH-cytochrome *c* reductase remained attached to microsomal membranes, as shown in Fig. 2.

Figure 5 shows the specific content of P-450, in terms of millimicromoles per milligram of protein, in the pellet after the microsomes had been incubated with Nagarse or Protease VII and centrifuged at 105,000 × *g* for 60 min. Incubation of microsomes with these proteases, even when performed anaerobically in the presence of 25% glycerol, caused degradation of the P-450, especially at high concentrations of the proteases. The results show that when microsomes of chick embryo liver cells are used as starting material, a concentration of 20 μg of Protease VII per milligram of microsomal protein appears capable of effecting substantial solubilization of cytochrome $b_5$ and NADPH–cytochrome *c* reductase with little degradation of cytochrome P-450.

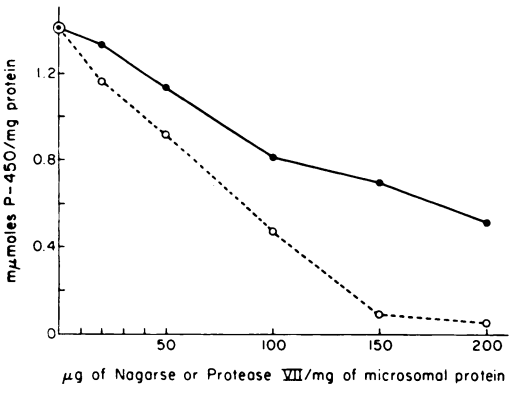


FIG. 5. *Specific content of P-450 in microsomes obtained from allylisopropylacetamide-treated chick embryos after treatment with Protease VII or Nagarse*

Experimental conditions were the same as described in the legend to Fig. 2. The specific content of P-450 in the pellets was calculated and expressed as millimicromoles of P-450 per milligram of protein. ●——●, Nagarse-treated; O- - -O, Protease VII-treated.

Based on these observations, P-450 was partially purified and solubilized from allylisopropylacetamide-treated chick embryo liver microsomes by incubating the microsomes first with Protease VII to remove cytochrome $b_5$ and NADPH–cytochrome *c* reductase, and then with Lubrol WX to solubilize P-450, followed by salt fractionation. The specific activity of P-450 in the final preparation was almost twice as high as that in the original microsomal fraction, as shown in the following section.

*Partially purified and solubilized cytochrome P-450 from chick embryo liver.* As shown in Table 1, the solubilized P-450 contained no cytochrome P-420, as measured by the CO difference spectrum, or cytochrome $b_5$. The activities of NADH– and NADPH–cytochrome *c* reductases were very low in the solubilized P-450 preparation; this was also indicated by the 76% loss of flavin, a prosthetic group of both NADH– and NADPH–cytochrome *c* reductases, from the microsomes.

The heme content of the preparation, determined by the pyridine hemochromogen method, was lower than the level of P-450 determined spectrophotometrically. The heme may be unusually unstable under the conditions of hemochromogen development, or the extinction coefficient of the solubilized P-450 preparation may be different from that of P-450 present in native microsomes. A similar discrepancy was observed by Miyake *et al.* (21) with their soluble P-450.

TABLE 1

*Comparison of enzymatic activities in solubilized P-450 preparation and original microsomes*

Chick embryos were treated with allylisopropylacetamide and soluble P-450 was prepared as described in MATERIALS AND METHODS. The contents of heme and flavin components and the activities of flavin-containing reductases in the soluble P-450 and original liver microsomes were measured as described in the text.

| Fraction | P-450 | P-420 | Cytochrome $b_5$ | Total heme | Total flavin | NADPH–cytochrome *c* reductase | NADH–cytochrome *c* reductase |
|---|---|---|---|---|---|---|---|
| | *mμmoles/mg protein* | | | | | *mμmoles cytochrome c reduced/mg protein* | |
| Microsomes | 1.69 | 0 | 0.454 | | 1.17 | 19.5 | 148.0 |
| Soluble P-450 | 3.0 | 0 | 0 | 2.63 | 0.284 | 1.47 | 5.88 |

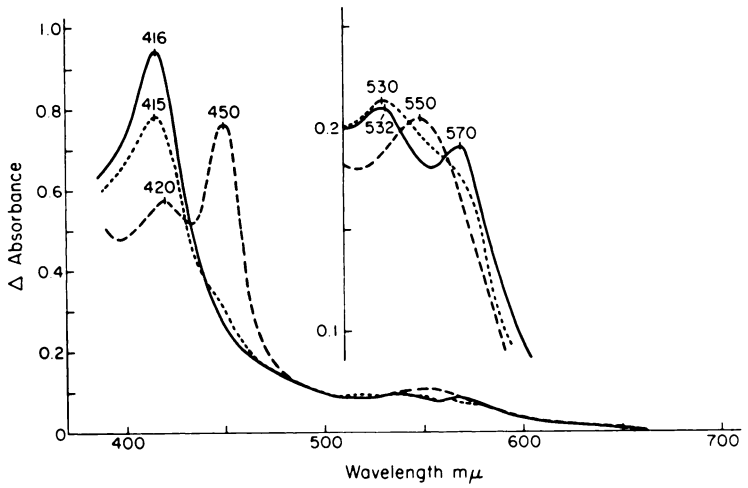


FIG. 6. *Absolute spectra of solubilized P-450 prepared from allylisopropylacetamide-treated chick embryo liver microsomes*

Chick embryos were treated with allylisopropylacetamide as described in the legend to Fig. 2. The solubilized P-450 preparation is described in MATERIALS AND METHODS. It was prepared at a concentration of 2.88 mg of protein per milliliter of 0.1 M sodium phosphate buffer, pH 7.0, containing 25% glycerol. The amount of P-450 was 2.21 mμmoles/mg of protein. – – –, oxidized spectrum (0.1 M sodium phosphate buffer, pH 7.0, containing 25% glycerol in the reference cuvette and solubilized P-450 preparation in the sample cuvette); -----, reduced spectrum (contents of the reference and sample cuvettes were the same as for the oxidized spectrum, except that the solubilized P-450 was reduced with a few crystals of sodium dithionite); – – –, carbon monoxide-reduced spectrum (contents of the reference and sample cuvettes were the same as for the oxidized spectrum, except that the solubilized P-450 was reduced with a few crystals of sodium dithionite and CO then was bubbled through it for 30 sec).

Figure 6 shows the absolute absorption spectrum of the solubilized P-450 from allylisopropylacetamide-treated chick embryo livers. The CO difference spectra of cytochrome P-450 in microsomes and of the solubilized P-450 preparation from allylisopropylacetamide-treated chick embryo livers are shown in Fig. 7. Although no peak at 420 mμ was detected in the CO difference spectrum of the soluble P-450 preparation, the absolute spectrum showed a peak at 420 mμ. The identity of the 420 mμ peak in the absolute spectrum is not known at present, but could be due to P-420 or hemoglobin.

*Induction of chick embryo liver microsomal P-450 with drugs and steroids.* As shown in Table 2, a 2–2.5-fold increase in liver microsomal P-450 content was found when 17-day-old chick embryos were treated with allylisopropylacetamide, 17α-hydroxy-11-ketopregnanolone, and etiocholanolone, all of which are strong inducers of δ-aminolevulinic acid synthetase (23, 24). The P-450 content of chick embryo liver microsomes was also enhanced by phenobarbital and 3-methylcholanthrene, although the induction of the hemoprotein was less than that obtained with allylisopropylacetamide or steroids. Cytochrome P-450 from 3-methylcholanthrene-treated chick embryos did not show the difference absorption maximum at 448 mμ in combination with carbon monoxide that had been observed in rats and rabbits (9, 10), even when the carcinogen was injected twice within 64 hr.

The CO–P-450 complex of this induced hemoprotein had a difference spectrum absorption maximum at 450 mμ, similar to that obtained in chick embryos treated with phenobarbital. On the other hand, when phenobarbital- and 3-methylcholanthrene-treated rat liver microsomes were used, the CO difference spectrum absorption maxima occurred at 450 mμ and 448 mμ, respectively, as reported by other workers (9, 10). The fact that it was not possible to demonstrate an induced P-448 in chick embryo liver was

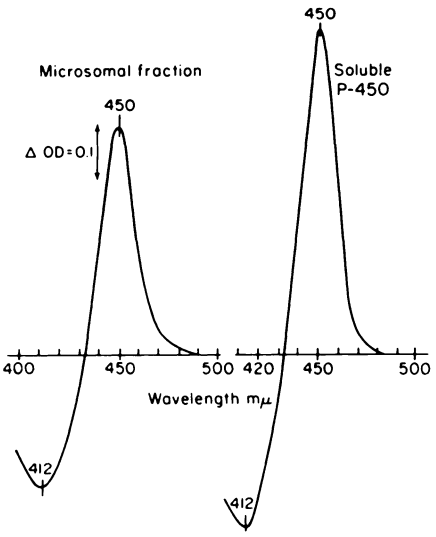


FIG. 7. *Carbon monoxide difference spectra of dithionite-reduced microsomes and soluble P-450 prepared from allylisopropylacetamide-treated chick embryo livers*

Chick embryos were treated with allylisopropylacetamide and microsomes and soluble P-450 were prepared from their livers as described in MATERIALS AND METHODS. The protein concentrations of microsomes and soluble P-450 were 2.5 and 1.9 mg/ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 25% glycerol, respectively. The CO difference spectra were determined by the method of Omura and Sato (27).

also confirmed by the ethyl isocyanide difference spectrum of this induced preparation. Imai and Sato (30) showed that an ethyl isocyanide–hemoprotein complex of liver microsomes had Soret peaks at 430 and 455 mμ, and that these peak heights were dependent on pH. Sladek and Mannering (11) showed that at pH 7.0 the peak height at 430 mμ was greater than that at 455 mμ when microsomes from untreated or phenobarbital-treated rats were used, but that when microsomes from 3-methylcholanthrene-treated rats were examined the 455 mμ peak was greater than the 430 mμ peak. As shown in Table 3, in our experiments at pH 7.0 the peak at 455 mμ of the ethyl isocyanide difference spectrum of microsomes from 3-methylcholanthrene-treated chick embryos was no higher than that at 430 mμ, the ratio of the 455 and 430 mμ peaks being similar to that obtained with

TABLE 2

*Effect of administration of drugs and steroids to chick embryos on liver microsomal hemoproteins*

Liver microsomes were prepared from 17-day-old chick embryos that had been treated with allylisopropylacetamide, 17α-hydroxy-11-keto-pregnanolone, etiocholanolone, or phenobarbital as described in MATERIALS AND METHODS. Other chick embryos received 3-methylcholanthrene at a dose of 3 mg/embryo once in 40 hr or twice in 64 hr, followed by incubation at 37°. The contents of P-450 and cytochrome $b_5$ were measured as described in the text.

| Treatment | P-450 | Cytochrome $b_5$ |
|---|---|---|
| | *mμmole/mg microsomal protein* | |
| Controls | 0.362 | 0.236 |
| Allylisopropylacetamide | 0.958 | 0.224 |
| 17α-Hydroxy-11-keto-pregnanolone | 0.705 | 0.256 |
| Etiocholanolone | 0.979 | 0.402 |
| Phenobarbital | 0.548 | 0.216 |
| 3-Methylcholanthrene | | |
| Controls | 0.444 | 0.303 |
| Once in 40 hr | 0.720 | 0.259 |
| Twice in 64 hr | 0.755 | 0.377 |

microsomes from untreated chick embryos. When the difference spectrum between the oxidized liver microsomal hemoprotein of allylisopropylacetamide-treated and untreated chick embryo was studied by the method of Kinoshita and Horie (31), the difference spectrum showed maxima at 575 mμ, 540 mμ, and 416 mμ (Fig. 8). This spectrum suggests that the P-450 of allylisopropylacetamide-treated chick embryo liver microsomes was spectrally similar to that observed with phenobarbital-treated rat liver microsomes (9).

*Substrate-induced difference spectra of solubilized P-450 from chick embryo liver.* Various types of compounds combine with microsomal cytochrome P-450 to give either type I or type II binding spectra (6–8). Studies were carried out to determine the binding capacities of partially purified chick embryo liver microsomal P-450 for certain natural steroids, which had previously been shown to be strong inducers of hepatic δ-aminolevulinic acid synthetase (23, 24). As summarized in Table 4, 17α-hydroxy-11-keto-

TABLE 3

*Ratio of ethyl isocyanide difference spectral peaks of microsomal P-450 derived from livers of chick embryos treated with allylisopropylacetamide, 17α-hydroxy-11-ketopregnanolone, and 3-methylcholanthrene*

Chick embryos were treated as described in Table 2. Microsomes were prepared and suspended in 0.05 M sodium phosphate buffer, pH 7.0, containing 25% glycerol, at protein concentrations of 2–3 mg/ml. Dithionite-reduced microsomes were placed in the reference and sample cuvettes; ethyl isocyanide (1.2 mM) was added to the sample cuvette, and the difference spectrum was recorded. Values represent ratios of the 455 and 430 mμ peaks of the ethyl isocyanide difference spectra.

| Treatment | Peak ratio |
|---|---|
| Allylisopropylacetamide | |
| Control | 0.143 |
| Treated | 0.095 |
| 17α-Hydroxy-11-ketopregnanolone | |
| Control | 0.229 |
| Treated | 0.161 |
| 3-Methylcholanthrene | |
| Once in 40 hr | |
| Control | 0.194 |
| Treated | 0.255 |
| Twice in 64 hr | |
| Control | 0.194 |
| Treated | 0.259 |

pregnanolone, a potent inducer of δ-aminolevulinic acid synthetase, caused no spectral change in P-450. On the addition of etiocholanolone, another strong inducer of δ-aminolevulinic acid synthetase, a very weak difference spectrum (type I) was recorded. Allylisopropylacetamide gave a type I spectrum. Induction of P-450 in chick embryo liver with allylisopropylacetamide or 17α-hydroxy-11-ketopregnanolone did not change the substrate-induced difference spectra. Aminopyrine produced a type I spectrum, and aniline and nicotinamide gave a type II spectrum, similar to those observed in other species (32).

## DISCUSSION

Miyake *et al.* (21) recently obtained a partially purified P-450 preparation by treatment of rabbit liver microsomes with Lubrol WX, an anhydrous condensation product of a long-chain fatty alcohol and ethylene oxide. Nishibayashi and Sato (17), in other studies, used Nagarse, a *B. subtilis* protease, to remove cytochrome $b_5$ and NADPH–cytochrome *c* reductase from microsomal membranes. Treatment of microsomes with either Lubrol WX or Nagarse resulted in little conversion of P-450 to its inactive form, P-420. The studies presented here show that another protease preparation derived from *B. subtilis*, Protease VII, is as effective as Nagarse in solubilizing cytochrome $b_5$ and NADPH–cytochrome *c* reductase from liver microsomal membranes. At a concentration of 20 μg of Protease VII per milligram of microsomal protein, cytochrome $b_5$ and NADPH–cytochrome *c* reductase were completely solubilized, and the specific content of P-450 remaining in the pellet decreased by less than 15%. If the ratio of Protease VII to microsomal protein was increased in the mixture, the P-450 loss increased, as measured by the decrease in its specific content (Fig. 5) The results presented above also show that NADH–cytochrome *c* reductase, in contrast to NADPH–cytochrome *c* reductase, was not readily released from the microsomal membranes (Fig. 2), in agreement with the earlier investigations of Ernster *et al.* (20), using deoxycholate, and of Orrenius *et al.* (33), using trypsin as solubilizing agents for cytochrome $b_5$ and NADPH–cytochrome *c* reductase. At a concentration of 20 μg of Protease VII per milligram of microsomal protein, cytochrome P-450 was not released to any appreciable extent into the supernatant fraction of Protease VII-digested mixtures. At higher concentrations of Protease VII cytochrome P-450 was released into the supernatant fraction as cytochrome P-420. It appears, therefore, that NADPH–cytochrome *c* reductase and cytochrome $b_5$ may be localized superficially in the microsomal vesicle membranes, whereas NADH–cytochrome *c* reductase and P-450 are present in the inner membranous structure (33).

We have also attempted to purify further the solubilized P-450 from chick embryo liver by chromatography on Sephadex G-25, DEAE-cellulose, and agarose columns and by sucrose density gradient centrifugation.

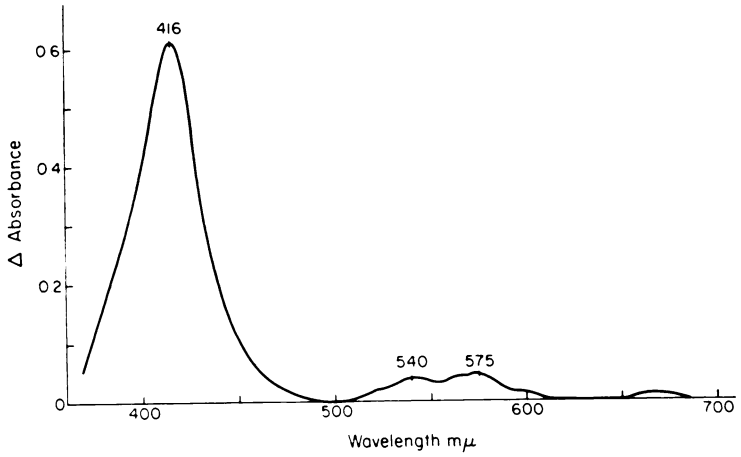


FIG. 8. *Oxidized spectrum of induced microsomal P-450 following treatment of chick embryos with allylisopropylacetamide*

Chick embryos were treated with allylisopropylacetamide and microsomes were prepared as described in the legend to Fig. 2. Liver microsomes were suspended in 0.05 M sodium phosphate buffer, pH 7.0, containing 25% glycerol. The oxidized spectrum was determined by the method of Kinoshita and Horie (31). The difference spectrum was recorded between the sample cuvette, containing microsomes prepared from treated chick embryos (10.1 mg of protein per milliliter, 0.958 mμmole of P-450 per milligram of protein, 0.244 mμmole of cytochrome $b_5$ per milligram of protein), and the reference cuvette, containing microsomes prepared from untreated chick embryos (5.4 mg of protein per milliliter, 0.357 mμmole of P-450 per milligram of protein, 0.282 mμmole of cytochrome $b_5$ per milligram of protein). Since the cytochrome $b_5$ contents in both cuvettes were nearly equal, the absorption of cytochrome $b_5$ is canceled out and the difference spectrum recorded is that of the oxidized P-450 induced by allylisopropylacetamide.

TABLE 4

*Substrate-induced spectra of soluble P-450 from chick embryo livers*

Chick embryos were treated as described in Table 2. P-450 was solubilized from liver microsomes of untreated and treated chick embryos as described in MATERIALS AND METHODS. Substrate-induced spectra of soluble P-450 were recorded after addition of substrates to the solubilized P-450 preparation (protein content, 3 mg/ml). The final concentrations of substrates in sample cuvettes were: steroids (in dimethylsulfoxide), 48 μM; aminopyrine (in dimethylsulfoxide), 15 mM; aniline (in $H_2O$), 15 mM; allylisopropylacetamide (in dimethylsulfoxide), 30 mM; nicotinamide (in $H_2O$), 30 mM.

| Substrate | Control | | Allylisopropylacetamide-treated | | 17α-Hydroxy-11-keto-pregnanolone-treated | | Spectral type |
|---|---|---|---|---|---|---|---|
| | Peak | Trough | Peak | Trough | Peak | Trough | |
| | mμ | | mμ | | mμ | | |
| 17-α-Hydroxy-11-keto-pregnanolone | —[a] | — | — | — | — | — | |
| Etiocholanolone | 400 | 420 | 410 | 420 | 410 | — | I |
| Progesterone | 420 | 390 | 420 | — | 415 | — | II |
| Aminopyrine | 400 | 420 | 400 | — | 400 | — | I |
| Aniline | — | — | 425 | — | 430 | — | II |
| Allylisopropylacetamide | 400 | — | 395 | 418 | 400 | 420 | I |
| Nicotinamide | 420 | — | 420 | — | 420 | — | II |

[a] Dashes mean that no spectral changes were detected under our experimental conditions when substrates were added to the sample cuvettes containing the soluble P-450 preparation.

The specific content of the P-450 could not be increased by any of these methods, partly because of its instability, since the hemoprotein changed readily to P-420 when subjected to column chromatography. The absence of cytochrome $b_5$ from the solubilized P-450 was also confirmed by the method of Alvares *et al.* (34), using DEAE-cellulose column chromatography accompanied by KCl gradient elution.

The procedure reported here for the solubilization and partial purification of microsomal P-450 from chick embryo liver has the following advantages: (a) the solubilized P-450 prepared by this method is suitable for measuring the absolute spectrum of cytochrome P-450, since no turbidity occurs with the solubilized preparation; (b) the procedure is somewhat simpler than that employing Lubrol WX, as reported by Miyake *et al.* (21); (c) the amounts of P-450 per milligram of protein are almost twice those in the original microsomes; and (d) the solubilized P-450 is devoid of cytochrome $b_5$ and shows minimal contamination with NADPH- and NADH-cytochrome *c* reductases and cytochrome P-420.

The preparation of the solubilized P-450 reported in this paper has some advantages over the techniques previously reported. Nishibayashi and Sato (17) used Nagarse for removal of cytochrome $b_5$ and NADPH-cytochrome *c* reductase but made no attempt at solubilizing the P-450 of their preparations. Furthermore, the total flavin contents of their P-450 particles and native microsomes were almost identical. The soluble preparation of Lu and Coon (4) contains at least 30% cytochrome P-420, as measured by the CO difference spectrum of the preparation eluted from the DEAE-cellulose column. The procedure of Miyake *et al.* (21) for rabbit liver microsomes involves passing the solubilized preparation through a Sephadex G-25 column followed by passage through a DEAE-Sephadex column. Their final preparation contains the same specific content of P-450 as native microsomes on a protein basis. Of the original P-450, only 5% was recovered in their final preparation. The solubilized P-450 preparation obtained by MacLennan *et al.* (22) from beef liver contains considerable amounts of cytochrome $b_5$ and P-420, although the specific content of cytochrome P-450 was increased 4–5-fold over that of the native microsomes.

The absolute spectrum of the P-450–CO complex of the solubilized preparation showed distinct peaks at 420 mμ, 450 mμ, and 550 mμ (Fig. 6). Since the CO difference spectrum revealed the absence of the 420 mμ peak due to P-420 or hemoglobin, the identity of the 420 mμ peak in the absolute spectrum remains to be elucidated.

Almost all the chemical agents and drugs which have been shown to be inducers of P-450 interact with P-450 *in vitro*; i.e., they cause substrate-induced difference spectra (32). For example, the addition of phenobarbital, aminopyrine, and fatty acids (32, 35) to rat liver microsomal preparations results in type I spectral changes. In the present studies allylisopropylacetamide was shown to be a type I substrate (Table 4), and the P-450 induced by this compound was similar to that induced by phenobarbital (Fig. 8). The oxidized form of the allylisopropylacetamide-induced P-450 showed absorption maxima at 416 mμ, 540 mμ, and 575 mμ, typical of a "low-spin" cytochrome P-450 (9). The addition of 17α-hydroxy-11-ketopregnanolone to the microsomes and to the solubilized P-450, however, did not result in the characteristic substrate-induced difference spectra, even after treatment of chick embryos with 17α-hydroxy-11-ketopregnanolone. This steroid is known to be a potent inducer of δ-aminolevulinic acid synthetase (23, 24), the rate-limiting enzyme of heme biosynthesis, and can increase P-450 content in the liver (Table 2) as well as the activity of certain model drug-metabolizing enzymes coupled to this "mixed-function" oxidase (36). The inability to detect difference spectra with the addition of 17α-hydroxy-11-ketopregnanolone to the P-450 preparation may have been due to the limits of sensitivity of the spectrophotometer employed in these experiments or to qualitative differences in the P-450 obtained from chick embryo liver as compared with that from other species.

More recently, Alvares *et al.* (37) have

reported a species difference in the induction of microsomal P-450 by 3-methylcholanthrene. Rats and rabbits treated with this agent showed a peak shift of CO-bound P-450 spectra to 448 mμ. However, treatment of mice and guinea pigs with 3-methylcholanthrene produced no peak shift in the CO-bound P-450 spectra. In the present experiments, 3-methylcholanthrene treatment of chick embryos caused a 1.6-fold increase in P-450 content; the CO-hemoprotein complex had an absorption maximum at 450 mμ, and no peak increase at 455 mμ over that at 430 mμ in the ethyl isocyanide difference spectrum was observed (Tables 2 and 3). These observations further confirm both qualitative as well as quantitative species variations in the induction of microsomal cytochrome P-450 by exogenous chemical agents.

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