THE DIFFERENTIATION OF RAT LIVER ENDOPLASMIC RETICULUM MEMBRANES: APO–CYTOCHROME P_{450} AS A MEMBRANE PROTEIN

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The proteins of washed microsomal membranes from adult rat liver were solubilized by 2% SDS and electrophoresed on polyacrylamide gels. Confirming earlier reports, a large Coomassie-Blue staining band in the ~ 50,000 MW region was identified as cytochrome P_{450} by four criteria: similar electrophoretic mobility to a purified cytochrome P_{450} preparation, an increase in this band after in vivo phenobarbital administration, a decrease in this band after in vivo allylisopropylacetamide administration, and direct specific binding of added purified heme to this region of a washed, unfixed gel. Although cyt P_{450} is not spectrally evident until just at the time of birth of the rats, a large band in this region was detectable in gels of microsomal membrane protein at all times, from three days before birth onward; this band also bound added heme after membrane proteins from fetal rat liver microsomes were electrophoresed on the gels. The conclusion was that apo-cyt P_{450} is present in microsomal membranes at these times during differentiation, and that, regarding this protein, during differentiation heme is bound to the apo-protein already there, concomitant with a synthesis of more cyt P_{450} molecules. The process of differentiation of this membrane type is also discussed.

INTRODUCTION

During development of mammalian hepatocytes there occurs a differentiation of the endoplasmic reticulum (ER) membranes; the experiments which support this idea deal with the in vitro measurements of the activities of microsomal enzymes which are known to be constitutive* parts of these membranes. Thus, it has been well documented (1–9) that the activity of the well-known marker enzyme of liver ER, glucose-6-phosphatase, is very low before birth in many species and rises dramatically soon after birth. Certain electron transport enzymes, which are partly or completely microsomal, such as the NADH- and NADPH-cytochrome c reductases and NADH-ferricyanide reductase, have a low activity in whole chicken embryo (10), in liver microsomes of newly-hatched chick (11), in microsomes of newborn rabbit (12), and in liver homogenates (13) or microsomes (8) of newborn rats. While cyt b_5 is present in hepatic microsomes of chicken embryos (14), it does not appear in rat liver microsomes until after birth (8). A low ability to synthesize ascorbic acid has been reported in liver microsomes of newborn rabbits and rats (15), and there occurs a deficiency in the early postnatal period of the synthesis of bilirubin

*We define a constitutive component as that component, mostly protein, but also lipid, which is an intimate part of the complete, functioning membrane structure, a component which resists removal from the membrane structure by mild means, which is held either firmly or loosely to the membrane, and whose presence in the membrane may or may not, however, be necessary for the existence of a membrane structure.

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glucoronides by microsomes of human, rat, rabbit, and guinea pig liver (12, 16, 17), as well as the transferase enzymes which are involved (18, 19). Drug-metabolizing enzymes (8, 20–25), and the associated cyt_{450} (8, 25), are very low in microsomes of newborn rabbits and rats and appear only after birth. Because the injection of actinomycin D inhibits the increase in activity with time of glucose-6-phosphatase and of NADPH-cyt c reductase and because puromycin also inhibits glucose-6-phosphatase (8), it was thought that the increased activity of these enzymes, at least, is due to the synthesis of the proteins, and not to an activation of already existing proteins (8). Based on this consideration, and on others, including the observation that an ER membrane structure exists (8, 9) even in the absence of demonstrable activity of many enzymes, it was postulated that a functional membrane, in this case the ER membrane, is produced in a multistep operation in which a structural framework is assembled first, and consequently the enzymes peculiar to the membrane are synthesized and inserted into a growing and differentiating mass, not all at once, but in a definitive, multistep sequence (8, 9). The data in this paper add further confirmation to the hypothesis, but with some modification, in that some of the structure of the precedent semifunctional membrane may be due to proteins which have to be activated or completed to express their activity, specifically in the case at hand, to cytochrome P_{450}.

METHODS

Animals

All rats were of the Sprague–Dawley strain, obtained from Holtzman Rat Farm, Madison, Wis., and were kept on an ad libitum Ralson Purina chow diet. Nonpregnant and pregnant female adults, weighing 200–250 gm, were used for all experiments except that male rats of the same size were used for the phenobarbital experiments. Pregnant rats were received on the 7th day after conception (as noted by the company) and kept on the same diet until used. The age of the fetuses was calculated from the delivery date of one of the pregnant rats, which was the 21st day ± 1 day after conception.

Preparation of Microsomal Fractions

Nonpregnant rats were killed by dislocation of the cervical column and the livers immediately excised. Pregnant rats were anesthetized by ether and the fetuses removed; livers from fetuses of the same age were pooled until about 1 gm wet weight was collected. Livers from adults were cut into small pieces and passed through a tissue press before homogenization, while livers from the fetuses were used as such. In the case of adult animals, 5 gm wet weight livers were homogenized with a motor-driven teflon homogenizer for 20 up-and-down strokes in 22 ml of 0.25 M sucrose. In the case of fetal livers, 1 gm of liver was homogenized in 7 ml 0.25 M sucrose. In both cases, the homogenate was centrifuged for 15 min at 10,000 × g (avg) to remove all the mitochondria and some microsomes. To separate rough from smooth microsomes derived from rough (RER) and smooth (SER) endoplasmic reticulum, a slight modification of the method of Dallner et al. (8) was used: the supernate from the 10,000 × g centrifugation was made 15 mM with regard CsCl, and 6.5 ml of this supernatant (equivalent to 1.6 gm wet weight liver in the case of the adult and ~ 1.0 in the case of the fetal livers) were overlayed on tubes containing a discontinuous

sucrose gradient (1 ml of 2.0 M sucrose; 3.5 ml 1.3 M sucrose). After centrifugation for 60 min at 160,000 × $g_{(avg)}$ in an angle-head rotor (A 321 of the International Centrifuge Co.), the free polysomes and glycogen if present were sedimented as a pellet to the bottom of the tube; rough microsomes ended up as a band at the 2.0 M:1.3 M sucrose interface, while smooth microsomes ended up as a band at the 1.3 M–0.25 M sucrose interface (8). The bands were collected separately by Pasteur pipette, made up to 5 ml volume with 0.25 M sucrose, and each suspension was mixed well with 5 ml of a solution containing 30 mM KCl and 20 mM EDTA, pH 7.4, in order to wash out cisternal contents, adsorbed proteins, and, in the case of the rough microsomes, to remove most of the ribosomes (26). Each mixture was then spun for 60 min at 105,000 × $g_{(avg)}$ to sediment separately the washed and extracted rough and smooth microsomes. The microsomal pellet, which consisted almost solely of membranes (26), was resuspended in a teflon homogenizer in various amounts of distilled water, so that the final protein concentration was approximately 10 mg/ml, as determined by the Lowry procedure (27).

SDS–Polyacrylamide Gel Electrophoresis

Membrane suspensions were solubilized by adding an equal volume of 4% sodium dodecylsulfate (SDS) containing 0.1 M Na_2CO_3 and 20% β-mercaptoethanol (cf. 28). The clarified suspensions were left overnight in a cold room and were centrifuged at low speed the next morning to sediment undissolved proteins; the amounts of protein still insoluble varied from 0 to 10% in the various experiments.

The gel system used was essentially the discontinuous buffer system as described by Neville (28). Cyclindrical tubes 10 × 0.5 cm were used. The stacking and separation gels contained 3.2% and 11.1% total acrylamide, respectively, and of the total acrylamide, 6.25% and 0.9% methylenebisacrylamide, respectively. The pH's of the lower reservoir and lower gel buffer, of the upper reservoir buffer, and of the upper gel buffer were, respectively, 9.18, 8.64, and 6.10. Electrophoresis was carried out for 8 to 12 hr at 0.4–0.6 mA/gel at room temperature, until the tracking dye, bromphenol blue, at a final concentration of 0.001% in the upper reservoir buffer, reached the bottom of the tube. This point was also marked in the heme-binding experiments (see below) by inserting a thin stainless steel wire into the gel at that level. Except in the heme-binding experiments, gels were fixed by keeping them in 50% methanol–7% acetic acid overnight at room temperature, and were then stained with a solution of 0.25% Coomassie Blue in 50% methanol–7% acetic acid. Excess dye was removed by extensive washing with 30% methanol. The washed gels shrink under this procedure, so that finally the gels were washed with distilled water and kept in water to allow expansion to the original size. They were then scanned at 550 nm with a Gilford spectrophotometer equipped with a gel scanner. The molecular weights of SDS–protein complexes were estimated from standards (rat serum albumin, 64,000; α-amylase, 45,000; chymotrypsinogen, 25,700; hemoglobin, 15,500; cyt c, 11,700) and corresponded with the data in (28). These standards were run in every experiment and molecular weight estimates were determined by using these standards, calculating R_f values, and were graphed as indicated in (28). In this manner, gel runs with varying mobilities, indicated by the bromphenol blue front, as in Figs. 2, 4, and 5, could be compared with each other. In a similar manner, the positions of the ~ 50,000 MW component in the unstained gels were determined in the heme-binding experiments of Figs. 4 and 7.

Heme-binding Experiments

After electrophoresis, the gels were not fixed but were washed free of excess SDS and of buffers at room temperature overnight in 4 to 5 changes of distilled water. Each gel was then incubated with gentle shaking for 3 hr at room temperature in 30 ml of a solution containing 3 mg purified hematin in 0.1 phosphate buffer, pH 7.4, or in some cases, in 0.1 M glycine–NaOH buffer, pH 9.0. The hemin was obtained and purified from rat blood by the acetone–acetic acid–$SrCl_2$ method of Labbe and Nishida as described by Falk (29); a requisite amount was dissolved in a minimal amount of 0.1 N NaOH before use. After incubation, excess heme was removed from the gels by repeated washings with distilled water. The resultant gels were scanned as above, but at 415 nm wavelength; in cases where a large amount of membrane protein was run on the gels, a brownish band was visible to the naked eye.

Miscellaneous

The concentration of cyt P_{450} in the various preparations was determined in the aqueous suspension of microsomes by the method of Omura and Sato (30). The purified cyt P_{450} was a gift of Dr. A. Alvares (Rockefeller University) prepared by the method of Lu and Levin (31).

The protocols for the injections of phenobarbital (PB) and of allylisopropylacetamide (AIA) are given in the legends to the figures showing the results of these experiments.

RESULTS

Electrophoresis of Membrane Proteins on SDS Gels

The ER membrane proteins were more easily solubilized in the SDS solution described in the Methods section than in any solution containing various proportions of phenol-acetic acid-urea; 2% seemed to be the optimum SDS concentration. Using this 2% SDS–0.05 M Na_2CO_3–10% mercaptoethanol solution, from 90–100% of the proteins were solubilized, as determined by the amount of precipitate after low-speed centrifugation, and as noticed by the absence of a stained band at the interface between the stacking and running gels after electrophoresis. Heating the SDS–protein solution for 15 min at 60°C had no effect on the solubilization, nor on subsequent electrophoresis. The addition of the Na_2CO_3 and mercaptoethanol made for greater reproducibility among the same samples placed on different gels. The presence of urea in the SDS solution, from 0.5 M to 2.0 M, had no effect on the subsequent electrophoretic pattern; indeed higher concentrations of urea gave patterns that tended to smear. After various trials, for better reproducibility and for greater separation and resolution of bands it was decided to use gels having 11% cross-linking. Buffers were also used in the manner previously described. The SDS–protein solution was able to be kept in the cold room for up to two weeks with no distortion of the subsequently run electrophoretic band pattern; after two weeks, the distinctiveness of the band pattern began to disappear, for unknown reasons.

Since isolated microsomes contain proteins entrapped in the vesicles, adsorbed proteins, like hemoglobin, and, in the case of rough microsomes, ribosomal proteins, it was necessary to wash out from the preparation those nonmembrane proteins. The washing

procedures used removed ~ 40% of the originally isolated rough microsomal proteins, ~ 10% of the phospholipids, and ~ 90% of the RNA; based on these values, about one-half of the washed-out proteins are ribosomal proteins. This washing procedure does not remove the more easily solubilized membrane proteins, such as cyt b_5 or the NADPH-cyt c reductase (26). Washing the microsomal preparation as recommended by Phillips and coworkers (32, 33), using 1.0 M NaCl, and 0.1 M Na_2CO_3, and 0.1 M $NaHCO_3$, gave gel patterns exactly the same as those obtained with the procedure given above. None of the large bands were found to move with the mobility of albumin or of hemoglobin run at the same time. Because of these findings, it is assumed that nearly all the bands, with the possible exception of some ribosome proteins in the ~ 25,000 MW region, represent constitutive proteins of the membranes.

Since the membrane frations contain about 40% phospholipid by weight, it is conceivable that some of the Coomassie Blue stained bands were phospholipid or that the phospholipids affected the migration of the proteins. However, the SDS does remove the phospholipid from the proteins, with the former migrating to the gel with the bromophenol blue marker (34), and, under the conditions of fixation, staining, and destaining, the phospholipid does not bind the Coomassie Blue dye (34). Furthermore, removing the lipid from the preparation by either 90% acetone or 2:1 chloroform–methanol did not affect the subsequent gel pattern; it did cause some sharpening of the bands, but no appearance of new bands or disappearance of original bands occurred (34).

There has been much work on the electrophoresis of solubilized ER membrane proteins from various sources, using either urea (35) or SDS (32, 36–42) electrophoresis. Our electrophoretic pattern (cf. Fig. 6, adult) is similar to some of these findings (38–41) in that the membrane proteins cover the range of molecular weight from ~ 15,000 to over 100,000. Except for a larger amount of material in the ribosomal protein region, the patterns obtained from rough microsomes were nearly identical to those obtained from smooth microsomes, confirming earlier work (32, 33, 42–44) on the protein* (and enzymatic) identity of these two membranes; differing results (37, 38) can be ascribed to cross-contamination of the preparations (cf. 33). The number and position of the bands from liver microsomal membranes of adult animals were greatly reproducible from experiment to experiment. However, there was one great variation, and that was the amount of the stained band at the position of about 50,000 MW (Fig. 3). This band, which is the major one of these membranes, can vary from ~ 10% (Figs. 3B, 6, 7) to ~ 40% (Figs. 1, 2, 3A, 4) of the material in the gels, as estimated from the areas under the scanned peaks. In the latter case, the pattern is very similar to that shown by Hinman and Phillips (32) and by Dehlinger and Schimke (41). The amount of protein stain in this band, which had been previously tentatively identified as the apo-protein of cyt P_{450} (41, 45) and which we have confirmed (see below), varied greatly from experiment to experiment, and we ascribe its variation to the in vivo induction of this cyt P_{450}. This variability in induction may have been caused by the spraying of pesticides in the animal room, and even by the kind of bedding used in the cages (46); we tried to control the variability by keeeping the animals under controlled conditions soon after they arrived from the breeding company, but with no success.

Identification of Major Band as Apo-Cyt P_{450}

The band at the MW region of ~ 50,000, which varied in intensity, as mentioned

*Kreibich, G. and Sabatini, D. (personal communication).

above, was identified as the apo-protein of cyt P_{450} by four criteria: similar movement on the gel to a purified cyt P_{450} preparation, the increase in this band by PB injection, the decrease in this band by AIA injection, and a direct in vitro binding of heme to the ~ 50,000 MW region of the gel after the electrophoresis of membrane proteins.

Comparison of banding patterns of membrane proteins to that of purified cytochrome P_{450}. Figure 1 shows a comparison of the banding of membrane proteins to that of a purified cyt P_{450} preparation in which the cyt P_{450} was purified 3- to 6-fold from the ER membranes (dependent on the amount of cyt P_{450} in membranes from "normal" animals). The pattern of the cyt P_{450} preparation is the same as that shown by Dehlinger and Schimke (41), but differs from that obtained by Levin et al. (45), where two equally staining bands, at 50,000 and 59,000 MW, were the major bands.* Since in the latter case the purified cyt P_{450} was obtained in the same way as was the gift preparation from Alvares and has about the same specific content, no explanation can be provided for this variation. Figure 2 shows a scan of a set of similar gels, where at high gel loads, other membrane proteins can be detected in the purified cyt P_{450} preparation (Fig. 2C), but only one symmetrical band is discernible, at about 50,000 MW, when the amount of cyt P_{450} preparation placed on the gel is lowered to 10γ, 5γ (Fig. 2E), or 2γ (Fig. 1). The major band in the ER membrane preparation was tentatively identified as cyt P_{450} since its electrophoretic mobility was similar to that of the major band in the cyt P_{450} preparation. It should be pointed out that there seems to be only one protein at this ~ 50,000 MW region, as can be seen from the sharpness and symmetry of the stained peak, when normal amounts of cyt P_{450} are present (Figs. 6, 7).**

Effects of PB and AIA injections on gel banding patterns. Since it is well known that the levels of cyt P_{450} in liver ER are responsive to drugs, particularly PB treatment [cf. review (47)], the effect of PB injection was tested. Figure 3 gives two typical results of representative experiments. When the amount of cyt P_{450} in liver ER is normal, 0.60–0.7 nmoles/mg protein [cf. (30)], the injection of PB increases its specific content about 3-fold (Fig. 3A) and 5-fold in terms of total liver amount (26). The amount of stained band in the ~ 50,000 MW region of the membrane seems to likewise increase (Fig. 3A); this effect was also found by Levin et al. (45). When the amount of cyt P_{450} is already high due to induction for unknown reasons, as mentioned above, the staining of this band is also high, and injection of PB does not increase the spectroscopically determined amount in the microsomal suspension, nor does it increase the amount of stained material in this region of the gel (Fig. 3B). Thus, by these criteria, the changes in this particular band mirror the changes in the amount of cyt P_{450}.

Another study, based on the observations of De Matteis (48), and confirmed by Levin et al. (49) and Satyanarayana et al. (50), that the injection of AIA, preferably with PB, causes a selective destruction of the heme of cyt P_{450}, not affecting the heme of the other ER membrane hemoprotein, cyt b_5, was confirmed here. We found that the AIA treatment causes a loss of nearly two-thirds the heme of cyt P_{450} in 1 hr, leading actually to a greenish coloration of the isolated microsomes. Figure 3B shows that concomitant with this loss there is a marked decrease in the ~ 50,000 MW band on the gel. While the results of this experiment could be confirmed once more, it was found that in three other cases, concomitant with a similar loss of spectroscopically determined cyt P_{450}, there was

*Welton and Aust (90) found two major peaks in this region in 1% SDS gels, at 45,000 and at 53,000 MW, differentiated through PB and 3-methylcholanthrene injections.

**However, partially confirming earlier work (45, 90), we also now find in purified cyt P_{450} preparations more than one bond, possibly four, in this region, using a better resolving gel system, namely SDS-slab gels having a linear 7.5 to 15% polyacrylamide gradient (A.P. Alvares and P. Siekevitz, Biochem. Biophys. Res. Comm., in press).

only little change in the stained band (Fig. 3A); this variation, which was not influenced by the dosage of AIA, nor by the duration of action of AIA, will be discussed in a later section. Nevertheless, it is felt that the results shown in Fig. 3B do provide additional evidence for identifying the ~ 50,000 MW band as cyt P_{450}.

Binding of heme to gel bands. Another way of proving that a band on the gel is a heme protein is to look for the presence of heme on the gel. Since Δ-aminolevulinic acid (ALA) is a specific precursor of microsomal heme (51), one Ci of ^{3}H–ALA was injected into a rat and 1 hr later the animal was sacrificed, microsomes obtained and washed, and the membrane proteins run on gels. When 120,000 CPM in 100γ protein were put on the gel, very little radioactivity could be obtained in any of the bands sliced from the gel, including the ~ 50,000 MW band, indicating the virtually complete removal of heme from the protein by SDS. This result, which confirms an earlier one (45), is not too surprising since the hemes of cyt P_{450} and cyt b_5 are noncovalently bound to the proteins, can be removed by 7 M urea (52), and have been shown recently to be also removed by SDS (45). A contrary finding has been found, that is, a retention on SDS gels of label from Δ-ALA injection (53); but this may possibly be due to a dissociation and reassociation of heme to proteins on the gel under the conditions of the experiment, as will be explained below. Schatz et al. (54) have found that by treating mitochondrial fractions with alkaline

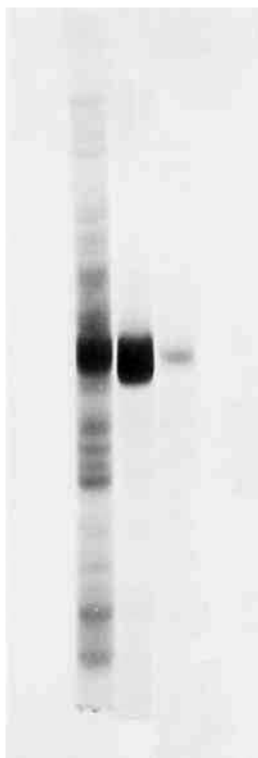

Fig. 1. Comparison of gels of proteins of smooth endoplasmic reticulum with purified cyt P_{450} preparation. The preparation of the membranes of the cyt P_{450} fraction is described in the text as is the SDS gel electrophoresis. 10γ of protein was run in the left and center gels, while 2γ protein was placed on the right gel. The cyt P_{450} contents of the membranes (left) was 1.85 nmoles/mg protein, while the cyt P_{450} preparation (center and right) had a content of 4.73 nmoles/mg protein.

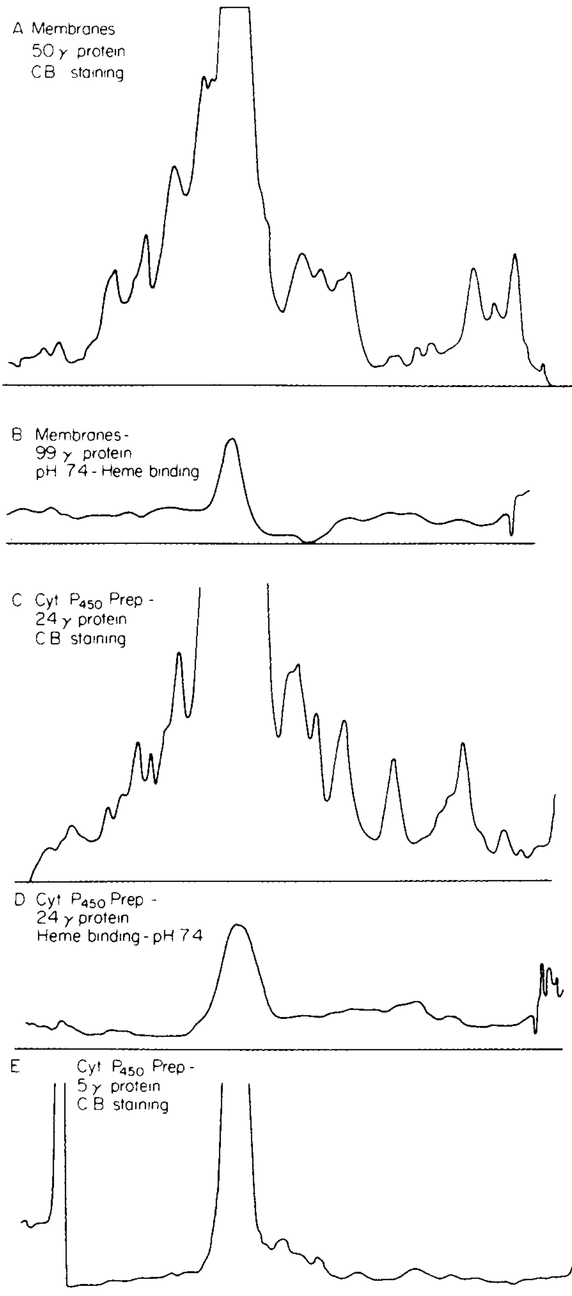

Fig. 2. Comparison of gel scans of smooth endoplasmic reticulum membrane proteins with purified cyt P_{450} preparation. The preparation of membranes and of the cyt P_{450} fraction is described in the text, as are the SDS gel electrophoresis and heme binding experiments. The cyt P_{450} content of the membranes was 1.85 nmoles/mg protein, while the cyt P_{450} preparation had a content of 4.73 nmoles/mg protein. In this and subsequent figures, CB = Coomassie Blue, and the amount of protein given is that amount placed on the gels. Also shown is the binding of heme when the gels were shaken at pH 7.4, as described in the text.

borohydride by the method of Takemori and King (55), they were able to covalently link the heme to the protein and in this way determine which one of the subunits was the heme-binding subunit in their cytochrome oxidase preparation obtained from these treated mitochondria. However, when this method was attempted on the microsomal preparation, still no retention on the gel of radioactivity after the injection of labeled Δ-ALA took place.

However, direct binding of heme to a nonfixed gel was done, as described in the Methods section. It was found that the pH was critical to demonstrate specificity of binding. Below pH 7, the heme came out of solution; between pH 7 and pH 8 there was specificity of binding, while at pH 9.0 all the proteins on the gel seemed to bind the heme, probably due to nonspecific binding to ionized groups of the basic amino acids. Figure 4 shows this effect in two experiments; at pH 7.4 there seems to be a binding of added heme to the ~ 50,000 MW region of the gel, while at pH 9.0 the other proteins took up the

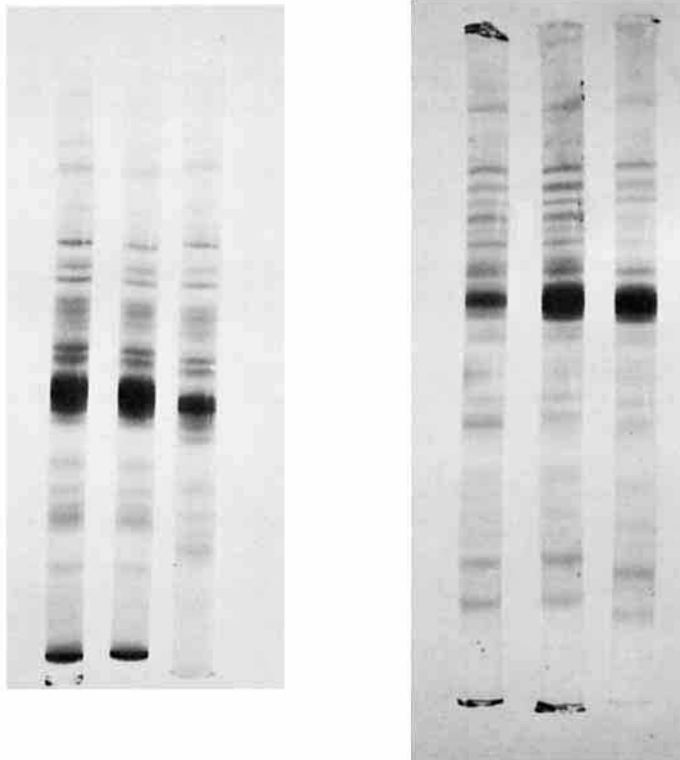

Fig. 3. Gel electrophoresis of smooth endoplasmic reticulum membrane proteins from normal, phenobarbital (PB)–injected and phenobarbital plus allylisopropylacetamide (AIA)–injected male rats. Male rats (~ 150 gm) were injected once a day for 5 days with 80 mg PB in 0.15 M NaCl/kg body weight each injection; they were sacrificed on the 6th day. In some cases, 200 mg AIA in 0.15 M NaCl/kg body weight was also injected 1 hr before sacrifice. Smooth membranes were prepared and the gels run as described. A. Right gel: normal animal, 43 μg protein, 0.95 nmoles cyt P_{450}/mg protein; center gel: PB–injected, 55 μg protein, 2.50 nmoles cyt P_{450} protein; left gel: PB plus AIA–injected, 57 μg protein, 0.90 nmoles cyt P_{450}/mg protein. B. Right gel: normal animal, 52 μg protein on gel, 2.35 nmoles cyt P_{450}/mg protein; center gel: PB–injected, 53 μg protein, 2.40 nmoles cyt P_{450}/protein; left gel: PB plus AIA–injected45 μg protein, 0.85 nmoles cyt P_{450}/mg protein.

heme also. Other experiments with heme binding are shown in Fig. 2, where the heme bound to the ~ 50,000 MW region of a membrane preparation (Fig. 2B) and also was bound to the region of the major band in the cyt P_{450} preparation (Fig. 2D). Figure 7 shows this binding once again onto a membrane preparation from adult liver and also from fetal liver membranes. When increasing amounts of ER membrane preparations from adult animals were placed on the gels, there was an increased amount of subsequently bound heme, but there was no strict quantitative correlation from the scan patterns between the CB-staining of the ~ 50,000 MW band and the heme binding at this region. The specificity of the binding was also examined. Figure 5 indicates that while the heme was bound at pH 9.0 to both albumin and amylase on gels, there was no binding at pH 7.4. The reason that the noncovalently bound heme protein, cyt b_5, shows no binding at the region of its 16,700 MW (56) is presumably (57) that the proportion of this protein in microsomal membranes is only a few percent, and the heme binding would hardly be detectable by our methods.

All of the above experiments would indicate that the major band on the gel which registers as a ~ 50,000 MW protein is the apo-protein of cyt P_{450}; this agrees with the earlier work of Dehlinger and Schimke (41) and of Levin et al. (45). A major staining band from ER membranes with a similar molecular weight, from 52,000–55,000, has already been observed by Hinman and Phillips (32), Schnaitman (37), Neville and Glossmann (39), and Black and Bresnick (53), with the latter also tentatively identifying it as the apo-protein of cyt P_{450}.

Banding Patterns of SDS Gels of Proteins of Liver Rough Endoplasmic Reticulum Membranes from Rats of Various Ages

Figure 6 shows the scans of gels after electrophoresis of proteins obtained from the liver ER membranes of rats of various ages. This pattern was obtained from one experiment in which the pregnant rats or the fetuses and young from this batch of animals were sacrificed at different times; other experiments gave similar results. The age span, from 3 days before birth to 4 days after birth to the adult, is precisely that when various enzymatic activities of these membranes are markedly increasing (cf. Introduction). Also, the rough ER was present all during this period of time, and that is why only rough microsome preparations were used, since smooth ER begins to appear only at birth (8, 9). Despite this change in enzymatic activities, the patterns obtained, particularly in the region of the gel, 30,000 MW to 100,000 MW, remain remarkedly constant. The only changes observed are in the lower molecular weight region of the gel, from 10,000 to 30,000. In the adult there is reproducibly found very little stainable material in this region, whereas in the fetuses and early young there is variation in the region; sometimes the pattern is such as that shown in Fig. 6 and sometimes not much material is found. The reason for this variation is not known; it could be due to ribosome proteins (MW ~ 25,000) in those cases where the washing did not remove the majority of ribosomes, or to degradation of proteins, though it was observed that there was no diminution of higher MW bands when these lower MW bands did appear. Nevertheless, it is remarkable that the pattern on the gels is much more constant than would have been predicted from the changes in enzyme activities.

Marked on the gel diagram of Fig. 6 is the position of a ~ 50,000 MW protein. Because of its position on the gel, and because at this position there is a binding of added heme, whether obtained from the adult membranes or from membranes of – 2 day fetus (Fig. 7), it is hypothesized that this band signifies the position on the gels of the

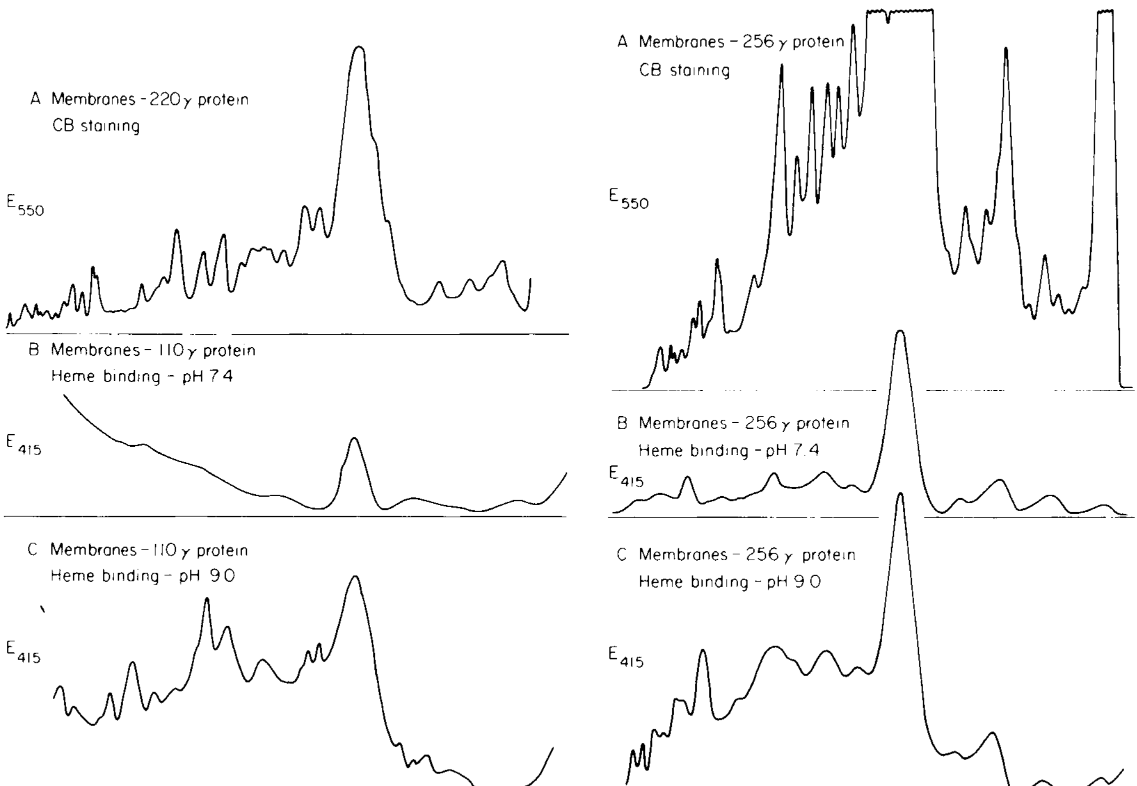

Fig. 4. Binding of heme to smooth endoplasmic reticulum membrane proteins on SDS gels. Description of the preparation of membranes, of SDS gel electrophoresis, and of the heme binding experiments are given in the text. The amounts of protein put on the gels are indicated on the figure. The membranes depicted on the lefthand graph contained 2.35 nmoles cyt P_{450}/mg protein, while those depicted on the righthand graphs contained 2.05 nmoles cyt P_{450}/mg protein.

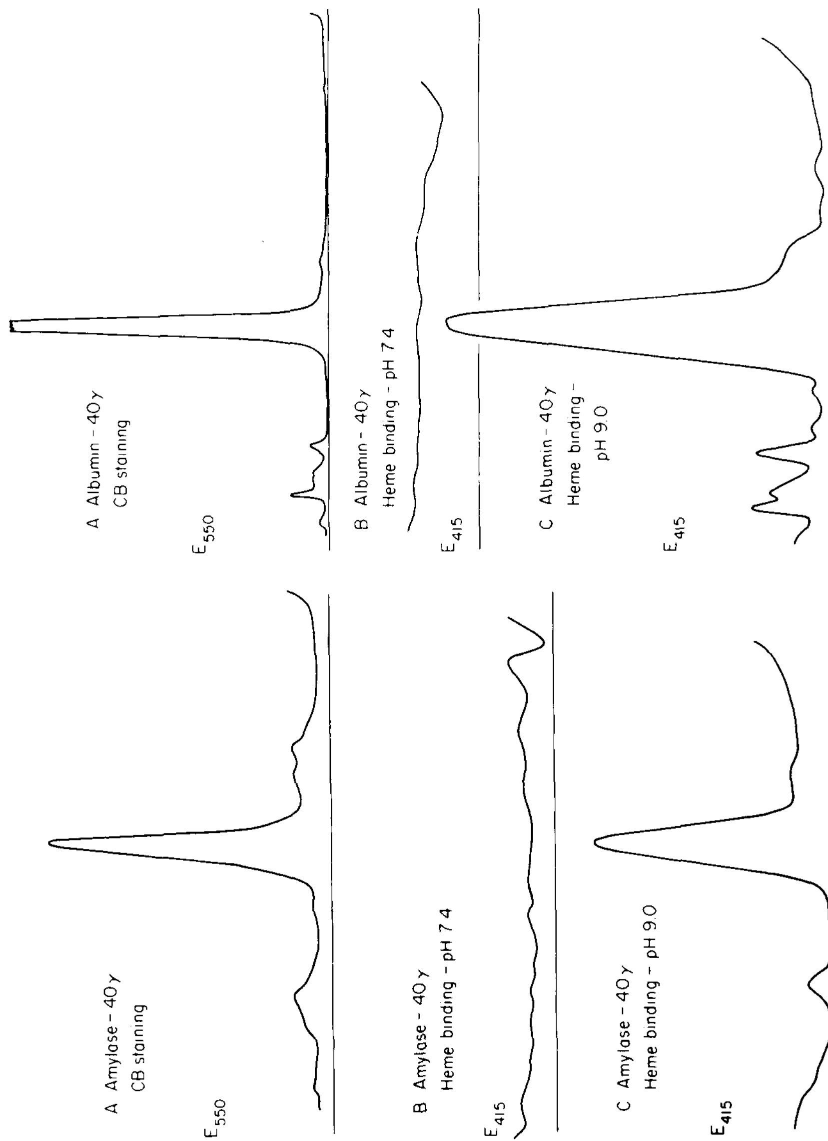

Fig. 5. Binding of heme to purified α-amylase and bovine serum albumin on SDS-gels. Description of the SDS electrophoresis and of the heme binding is given in the text. 40γ of the requisite proteins were placed on each gel.

apo-protein of cyt P_{450}. The resolution of this band would indicate that only one protein is contained therein, though of course other proteins of the same MW could contribute to it. It can be estimated that from – 3 days to the adult the amount of the stain of the band increases about 2-fold. Yet, as can be seen in the legend to Fig. 6 and from the data in (8), the spectrophotometrically detectable amount of cyt P_{450} increases at least 5-fold, and even much more since we cannot detect very small amounts of this cytochrome before birth. Also, because in the fetus we cannot detect cyt P_{450} by spectrum but do find heme binding in the ~ 50,000 MW region (Fig. 7), we come to the tentative conclusion that the apo-protein of cyt P_{450} exists in these membranes and only soon after birth is heme attached to it to give a spectrally identifiable heme protein.

DISCUSSION

The results point to the conclusion that during differentiation of the ER membranes, at least one protein, cyt P_{450}, is present in an inactive form, without its heme moiety, and that differentiation consists, in part, of the attachment of heme to the apo-protein concomitant with an increase in the proportion of this protein in the membranes. A similar conclusion has been reached for this cytochrome based on preliminary evidence (53).

It has also been inferred by immunological means (58, 59) and protein synthesis inhibition studies (60) that apo-cytochrome oxidase is present in membranes of mitochondria in derepressed yeast, but in this case it could be that one of the subunits (54) of this enzyme is absent. However, it has been shown (61) that heme can be added in vitro to cytochrome oxidase-deficient yeast mitochondria, resulting in some increase in activity indicating again the presence of an apo-cytochrome oxidase in these membranes.* Using immunological techniques, Raftell and Orrenius (62) found that they could detect cyt b_5 two days before birth in rat liver microsomes, at a time when spectrally none was detectable; perhaps again only the apo-protein was present. Even in the fully differentiated membrane in the adult, it has been inferred by "radioactive chasing" experiments that there exists a small pool of apo-cyt b_5 molecules in ER membranes (63, 64). These findings, showing the existence of an apo-protein free of its heme prosthetic group, should not be too surprising in view of the many experiments dealing with the easy exchange of heme between hemoglobin molecules (65), between hemoglobin and albumin (66), and between a soluble cyt P_{450} from Pseudomonas and apo-myoglobin (67). It should be pointed out that heme can bind to albumin (65) with propionyl groups of the heme (68) binding to the histidyl groups on the proteins (69), but under the conditions of our gel

*When washed rough or smooth microsomes of cholate-solubilized microsomes, from either fetus or adult rat livers, were incubated at room temperature with heme at pH 7.4, no increase in spectrally determinable cyt P_{450} was discernible. The results were the same whether varying amounts of heme were added, whether the incubating medium was varied, or whether the microsomes were centrifuged after the incubation, resuspended, and spectrally scanned for cyt P_{450}. One difficulty was the failure to wash out all of the added heme from the microsomes, with salt and EDTA washes varying from 10 to 200 mM. This failure is in contrast to the report (91) that heme added to an ammonium sulfate cut of supernatant from cholate-solubilized microsomes gave an increase in cyt P_{420}, the denatured form of cyt P_{450}; this result could be an indication of the existence of some apo-protein in adult microsomes or else the release of heme from already existent cyt P_{450} by the solubilization and purification procedures. Thus, by the above methods, we were unable to show the existence of apo-cyt P_{450} in microsomes from either fetal or adult livers. Using similar methods, Negishi and Omura (63) were also unable to detect spectrally apo-cyt b_5 in microsomes from adult rat livers.

experiments, no binding takes place (Fig. 5). While there seems to be some coordination between the biological synthesis of the protein and of the heme, as in the case of tryptophan pyrrolase (70), cyt c (71), and of cyt P_{450} (72), this is not necessarily so, since protein and heme synthesis can be separated in time (73), the heme and protein moieties of microsomal cyt b_5 exhibit different turnover rates (74), and also the hemes of the two microsomal cytochromes, b_5 and P_{450}, are turning over at different rates (75).

What is the relationship of the apo-cyt P_{450} to the other components of the ER membrane? One possibility for the lack of the completed heme protein in the ER membranes

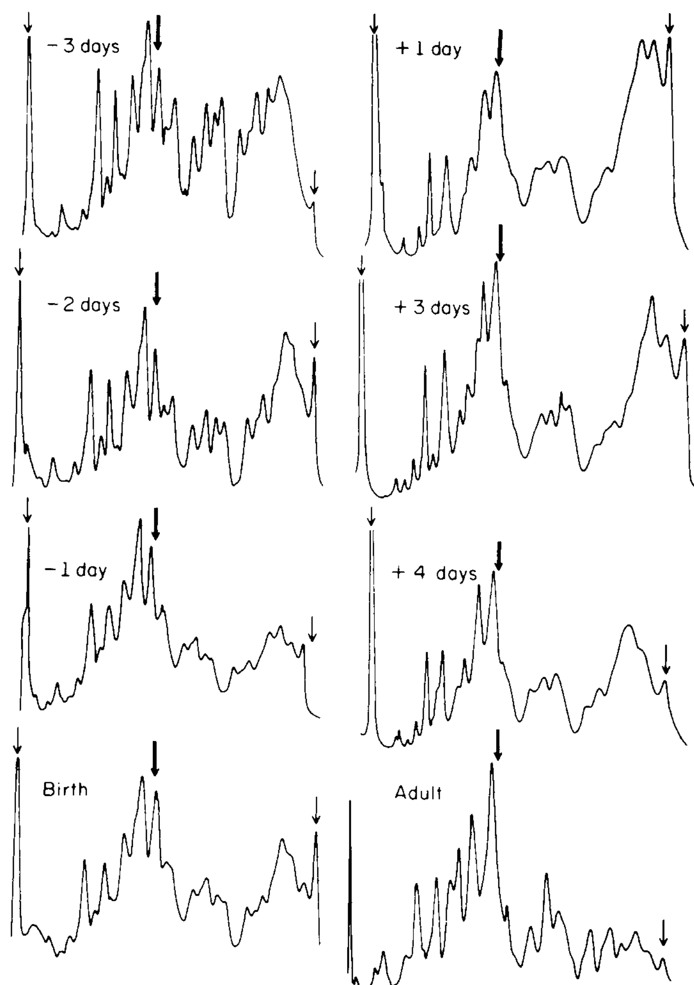

Fig. 6. Scans of Coomassie Blue stained bands on SDS gels of liver rough endoplasmic reticulum membranes from rats of various ages. The preparation of membranes from rough endoplasmic reticulum and the separation of protein bands on SDS gels is given in the text. The amounts of protein on the gels varied from 53 to 65 μg. The cyt P_{450} concentration (nmoles/mg protein) were: −3, −2, −1 before birth and at birth, undetected; +1 day, 0.15; +3 days, 0.30; +4 days, 0.35; adult, 0.75. The single arrows refer to the position of the stacking gel (left) and the band marking the bromophenol blue penetration (right); the double arrow marks the position of ~ 50,000 MW protein.

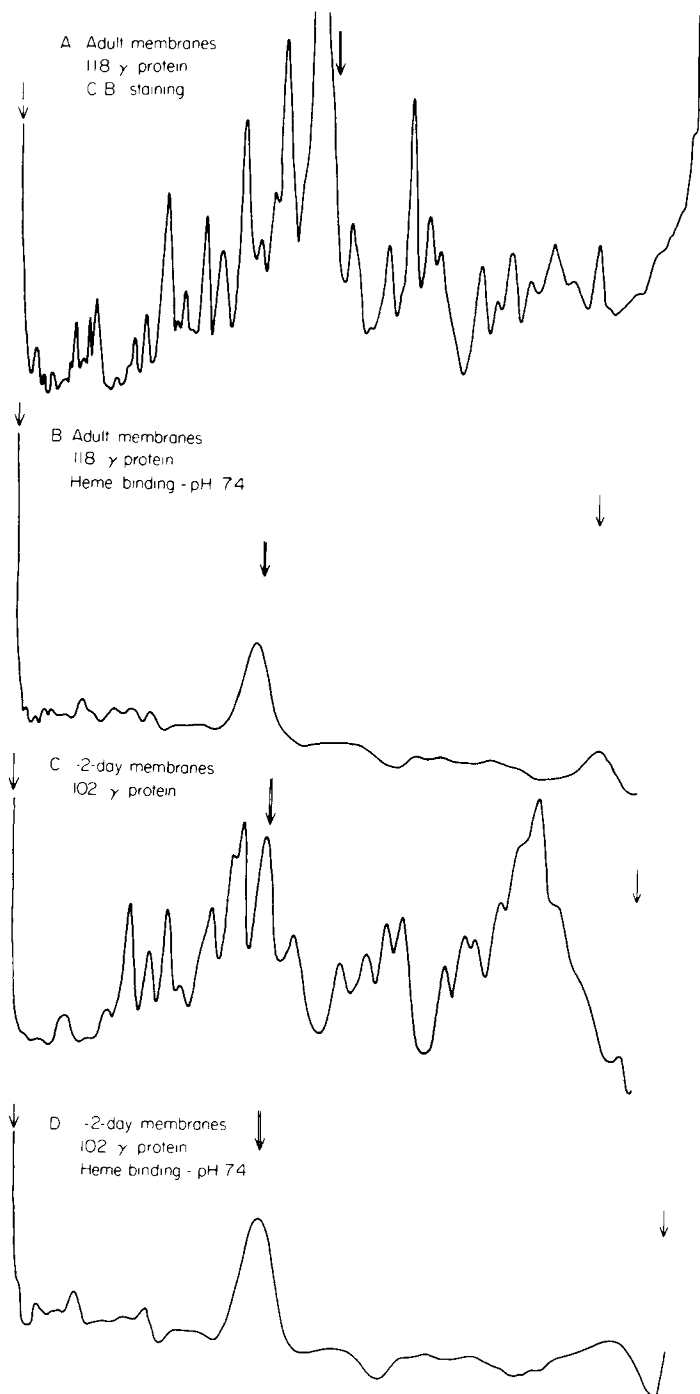

Fig. 7. Comparison of adult and fetal liver rough endoplasmic reticulum membrane proteins. The preparation of the membranes, of the SDS gel electrophoresis, and of the heme binding experiments are described in the text. The adult membranes came from the mother of the −2 day old fetus. Cyt P_{450} was not detectable in the fetal liver membrane preparation, while that of the adult liver contained 0.70 nmoles/mg protein. (A) and (C) – CB staining; (B) and (D) – heme binding. The double arrow shows the position of a 50,000 MW protein, while the single arrows indicate the top (left) and marker bottom (right) of the running gels.

before birth is that heme synthesis does not occur until birth, when for the first time we note spectral evidence for the existence of both cytochromes b_5 and P_{450} (8). However, these fetal rat liver cells can synthesize heme since mitochondrial cytochrome oxidase activity, while having in the fetus only about one-half the specific activity value of the adult, is still there in large amounts (76–78) and spectrally, both cytochrome oxidase (79) and cytochromes b and c (80) are also present in fetal rat liver mitochondria. Thus, it should be assumed that newly synthesized heme is available to the apo-cyt P_{450} in the ER membranes, particularly since some of the enzymes involved in heme synthesis are soluble as well as mitochondrial (81–83). Thus, while heme synthesis could still be limiting in the fetal rat liver, it also may be that the apo-protein is so structured within the membrane that it is unaccessible to available heme and that sometime during the perinatal period, its arrangement is so altered within the membrane that it is able to accept available heme, and thus functions in electron transport. Under certain conditions, the apo-protein can exist as a membrane protein, as during development (Figs. 6, 7) and even after heme destruction (Fig. 3A). Also, sometimes under the same conditions the removal of the heme seems to loosen up the relationship of the apo-protein to the membrane and it is lost (Fig. 3B).

It is intriguing to compare the calculated relative amount of cyt P_{450} in ER membranes from adult animals, as determined spectrally and from the gel pattern. Under conditions of induction by PB, the gel patterns show that the CB-stainable band at ~ 50,000 comprises some 40% of the CB-stainable membrane proteins (cf. Figs. 3, 4). Assuming a MW of 50,000 and assuming that the band at ~ 50,000 MW is all cyt P_{450} [which may not be true, see (45, 90)], and assuming the correct absorption coefficient for cyt P_{450} based on the hemochromogen [cf. (30)], it can be calculated that only from 10–15% of the membrane protein is cyt P_{450}. Similar calculations for the normal animals show a similar discrepancy. It is unlikely that the presence of totally different proteins in the ~ 50,000 MW region can account for such a large discrepancy; thus one possible conclusion is that even in the ER membranes from adult liver a good deal of the cyt P_{450} is present as the apo-protein in an amount much greater than the relative amount of apo-cyt b_5 molecules in these membranes (63, 64).*

Whether the apo-cyt P_{450} can be considered to be a structural protein is a moot question if by structural protein we mean those proteins whose presence is necessary for a structure to exist. It can be extracted and purified from the ER membranes only in the presence of detergents (31), indicating hydrophobic interactions with other membrane components; but the purified preparation contains very little phospholipid (31) and, as can be seen from Figs. 1 and 2, no other major protein bands. We do not know whether an ER membrane structure can exist without apo-cyt P_{450}, but we know, from the function of the active protein, that it must interact with oxygen, with substrates, with one and

*However, the seeming paradox mentioned above may be resolved as a result of a recent paper (92) in which a purified cyt P_{450} preparation from rat liver, having a specific content of ~ 2 nmoles/mg protein, was stated to have a sedimentation coefficient of ~ 18S, with a presumptive MW of ~ 350,000. Thus, SDS treatment of such a preparation could result in the appearance of subunits with a MW of ~ 50,000. Recent work by Welton and Aust (90), confirmed recently here by running purified cyt P_{450} preparations on gels of a cross-linking gradient, does indicate that the ~ 50,000 MW region does contain 4–5 bands, some of which are differentially induced by PB and methylcholanthrene. If the membrane-bound cyt P_{450} contained six subunits, with only two having functionally bound heme and the others being substrate-activating subunits, then the amount of cyt P_{450} in the membranes as determined spectrally would agree with that estimated amount as determined by the gel pattern, and the discrepancy would be resolved.

possibly two flavoproteins, and possibly with cyt b_5 (84), and because of the need for detergents to solubilize it, also with phospholipids. Because of these characteristics, and because in some cases it reaches high proportions in the ER membranes, it has been called a structural protein (32). However, the great variability in the concentration of this seemingly single protein would suggest that there are additional binding sites, perhaps up to 4-fold, in the ER membrane for cyt P_{450} molecules, and these are enzymatically active binding sites, as induction experiments would indicate (cf. 47). A similar conclusion has been reached by showing that additional binding sites, up to 10-fold, exist for cyt b_5 molecules added in vitro to ER membranes (85, 86), and that these extra bound cty b_5 molecules are as active as the native, already existent hemoprotein (85, 86). This cyt b_5 is another protein the entire molecule of which requires detergent treatment to extract it from the ER membrane (87, 88). Since proteolytic treatment of the membrane removes an active protein, leaving behind a hydrophobic residue (85–88), it is probable that cyt b_5 is bound hydrophobically to other membrane components. Thus, in these two cases we have examples of hydrophobically interacting constitutive membrane proteins whose concentrations in the membrane can be increased manyfold. In the case of cyt P_{450} we have a major membrane protein which on the one hand acts like a structural protein having distinctive functional properties, and on the other hand exhibits a property, greater variations in concentration, which we would expect of proteins not intrinsic to membrane structure. Therefore, to that finding (8) of constantly changing proportions of membrane enzymes, we can now add that even those functional proteins, which like cyt P_{450} have a structural role, can also occur in membranes in varying proportions to the other proteins therein.

In summary, membrane differentiation seems to consist both of a synthesis of new membrane proteins, as summarized in the Introduction, and a completion, in a sense, of proteins already there in the membrane. The increase in membrane mass and its differentiation seem to occur by three processes: the additon of newly synthesized proteins similar to those already there, the addition of newly synthesized proteins unlike those already there, and the activation or completion of protein molecules already present. During that period of time of differentiation (cf. Introduction), the ER membrane exists as a recognizable and collectable entity, but its basic functioning and its character are changing as a result of the above processes. A similar conclusion has been reached regarding the mitochondrial inner membrane (89). A corollary to these findings is the hypothesis that during phylogeny structures are formed from specific aggregating molecules and their function is in a sense added on. It is conceivable that what we now observe as complete electron transport membranes, as in ER, or energy transducing membranes, as in mitochondria, chloroplasts, or chromatophores, were evolved from structures which did not have this function, or perhaps had no function at all. It is not that electron transport proteins came together to form a structure because it was more efficient for overall function to do so, but that existing structures were modified to serve this purpose. It may be said that life evolved from structures acquiring functions.

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