Biochimica et Biophysica Acta, 462 (1977) 671–688

BBA 47395

CHARACTERIZATION OF MICROSOMAL ELECTRON TRANSPORT COMPONENTS FROM CONTROL, PHENOBARBITAL- AND 3-METHYLCHOLANTHRENE-TREATED MICE

III. IMPROVED RESOLUTION AND QUANTITATION OF MAJOR COMPONENTS IN AMMONIUM SULFATE FRACTIONS FROM TOTAL LIVER MICROSOMES

ROBERT H. MULL, MARGARET SCHGAGULER, HANS MÖNIG, THOMAS VOIGT and KURT FLEMMING

Institute for Biophysics and Radiobiology, University of Freiburg, Albertstrasse 23, D-7800 Freiburg i. Br. (G.F.R.)

(Received March 4th, 1977)

SUMMARY

Quantitation of microsomal components in ammonium sulfate fractions using a high-resolution sodium dodecyl sulfate-polyacrylamide gel electrophoresis system, and a comparison of these results with those from similar experiments on total liver microsomes has enabled us to identify and better characterize the interactions between microsomal electron transport components.

It was found that: (1) phenobarbital decreased the amount of one protein component of approximately 50 000 molecular weight while increasing a component of very similar molecular weight; (2) only two proteins appeared to be associated with CO binding; (3) another protein of approximately 68 000 molecular weight, one of the glycoproteins found in liver microsomes, appears to be induced by phenobarbital pretreatment; (4) the induction of NADPH-cytochrome *c* reductase activity after phenobarbital pretreatment is not dependent on an increase in the known NADPH-dependent flavoprotein, but rather on the increase in some component found predominately in our most soluble sub-microsomal fraction.

A very good separation of the above components was achieved by ammonium sulfate fractionation, e.g. simply on the basis of their solubility. This and the fact that the more-or-less soluble proteins were induced by phenobarbital or 3-methylcholanthrene respectively indicate that the solubility of membrane proteins plays a major role in the structure and function of microsomal membranes.

Abbreviation: SDS, sodium dodecyl sulfate.

I. INTRODUCTION

Liver microsomes, while not so easily obtained as erythrocyte "ghosts" or mitochondria, are obtainable in relatively large quantities. They are very complex but more than half of the protein is connected with microsomal electron transport [1, 2, 3]. They also have a major advantage for membrane functional studies in so far as their function and protein composition can be easily changed by pretreatment of animals with phenobarbital or 3-methylcholanthrene [4, 5].

The first major attempt to analyse microsomes in their functional subunits was made by McLennan [6], but these efforts were hampered by the inability to solubilize and resolve the membrane proteins from the fractions obtained by various methods. These experiments were followed and refined by the work of Lu and Coon [7, 8]. Finally, with the introduction of SDS-polyacrylamide gel electrophoresis by Weber and Osborn [9], the tools for an exact analysis of microsomal membrane induction were present.

II. METHODS AND MATERIALS

Microsomal preparation: Microsomes were prepared as previously described [3]. The number of animals for a preparation was limited on the one hand by perfusion and preparation time to a maximum of one hundred. Microsomes could be prepared from these animals in about 6 h, and yielded approximately 2 g of microsomal protein. This in turn yielded enough material, even in the smallest of our fractions, for extensive characterization of lipid and protein. On the other hand, a minimum number of animals, approximately ten $\widehat{=}$ 200 mg microsomal protein, was necessary for fractionation with a minimum of protein in the smallest fractions.

Ammonium sulfate fractionation: The microsomes were solubilized by the method of Lu and Levin [10]. This solubilized lipoprotein suspension was then salted out with increasing ammonium sulfate concentrations as previously described [1]. This procedure yielded five fractions in two steps. The fractions were designated by the step, I or II, and the range of ammonium sulfate used for the precipitation in per cent. The above procedure had a yield of 80–90 per cent for protein with only 10 per cent of microsomal lipid still present. The fractions appeared to be soluble in 50 mM phosphate buffer, pH 7.7, without cholate after precipitation, however, approximately 50 per cent of I 0–30 and 10 per cent of I 30–40 could be sedimented after dialysis in the presence of 0.1 per cent cholate. This was probably due to aggregation of the protein during dialysis. No aggregation was seen in fraction I 40–50, thus the tendency to aggregate decreases with increasing solubility as expected. Only fraction I 30–40 was precipitated in the second step, yielding fractions II 0–30, II 30–35 and II 35–43.

SDS-polyacrylamide gel electrophoresis: A recent review on the significance of this method in membrane studies [11] makes it unnecessary to recount details. However, varying the diameter or length of gels is a simple method for separating large quantities of proteins or achieving better resolution by extending the running time. The only problem encountered by this change was the formation of air bubbles in the gels. This problem can be countered by using the smallest possible amount of ammonium persulfate possible and de-aerating the gel mixture.

It should be noted here that, despite the optical impression of purity, this method is not capable of separating various proteins which have the same or similar molecular weights. This has recently been demonstrated by the separation of a "single" band from SDS electrophoresis into four components having different isoelectric points [12]. A short review of the many problems and limitations of SDS-polyacrylamide gel electrophoresis has recently been published by Maddy [13].

Our electrophoretic method was based on that of Weber and Osborn [9] with some modification [2].

Quantitative scanning of gels was based on the method of Matthieu and Quarks [14]. No absolute determination of protein is possible with this method as different proteins have differing affinities for Coomassie blue and the variations from experiment to experiment are large. One can, however, obtain a good relative protein composition expressed as per cent of total protein in any given experiment. The reproducibility of this method in our hands has already been demonstrated for total microsomes [2].

Labelling studies: The labelling of protein in control, 3-methylcholanthrene and phenobarbital pretreated animals was carried out with the injection of 25 μCi L-[1-^{14}C]leucine per animal, using 10 animals per pretreatment. L-Leucine was added to the radioactive sample, so that the final amount of L-leucine injected was 50 μmol/kg body weight. This injection was carried out intraperitoneally in a 0.2 ml volume of 0.9 per cent NaCl. Phenobarbital and 3-methylcholanthrene pretreated animals were induced as previously described, but [^{14}C]leucine was injected 24 and 12 h respectively after the last dosis of inducer, i.e. at half-maximal induction where induced proteins are still being synthesized, all animals being sacrificed 4 h later. Despite the large marker dosis used, the microsomal proteins were not adequately labelled to give sufficient counts using gels with a diameter of 5 mm. As much as 2.0 mg of labelled proteins had to be applied in order to receive 1000 cpm for the major components. This required the use of semi-preparative SDS gels, 7 cm long × 1 cm diameter. This method was based on that described by Dehlinger and Schimke [15]. Running time was 24 h, 8 mA per gel. Gels were cut into 1 mm slices, dissolved with 30 per cent H_2O_2 and hyaminhydrochloride [16] and counted in a scintillation fluid as described by Carey and Goldstein [17].

Cytochrome c reductases: The ideal protein concentration for microsomal preparations was found to be 0.1 mg per ml for the NADPH and 0.01 mg per ml for the NADH dependent reduction of cytochrome c. However, fractionation of the NADH dependent reductase decreases its activity [18] so that 0.1 mg per ml protein was required in measuring this activity in our fractions. Otherwise, this method was as previoulsly described [1].

Electron spin resonance (ESR) studies: ESR observations were made with a Varian Associates X-band Spectrometer V-4502 employing 100 kHz modulation. The substances were frozen in quartz tubes. The ESR measurements were carried out at 77 K, using a liquid nitrogen dewar or the Varian Associates varibale temperature accessory without operating the heater. The latter equipment leads to a better signal to noise ratio, the standard error for the relative radical concentration in a single determination being ± 10 per cent. The recorded spectra were the first derivatives of the actual absorption curves. For quantitative measurements the peak to peak signal amplitude was used. The magnetic field strength was measured with a

Varian Associates proton resonance probe whose frequencies were determined by a Hewlett-Packard 524 electronic counter. The position of ESR spectra was obtained by an additional measurement of the microwave frequency with a Hewett-Packard frequency meter, model X 532 B.

Photometric determination of heme was by the method of Appleby [19]. All other methods were as previously described [1, 2, 3].

Materials: L-[^{14}C]leucine was obtained from Amersham Buchler, Braunschweig, G.F.R. and had a specific activity of 62 μCi/mmol. The D-leucine content was 0.1 per cent. Hyaminhydrochloride (hydroxide of hyamine) was obtained from New England Nuclear, Boston, Mass., USA. All other chemicals were reagent grade and commercially available.

III. RESULTS AND DISCUSSION

Microsomes and small molecular weight (< 40 000) components

Fig. 1 demonstrates the resolution (band number-top), and quantitation (band as per cent of total protein-bottom) of the major microsomal proteins as previously described [2]. In the present publication, however, we found it difficult to tabulate these data due to the large variations seen from fraction to fraction.

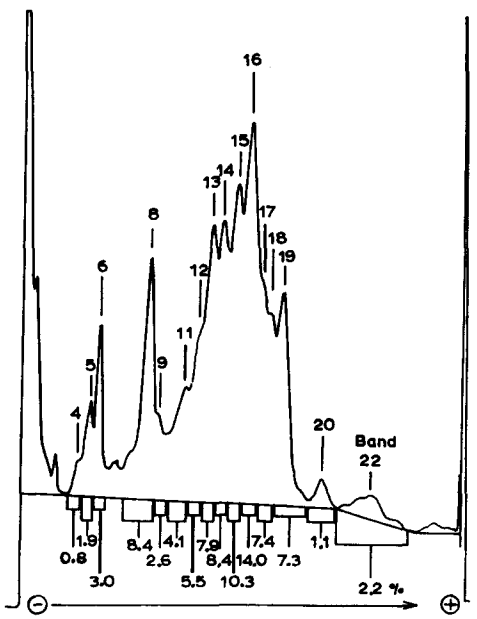

Fig. 1. Resolution and quantitation of major high molecular weight components in total liver microsomes from phenobarbital pretreated mice. The above scan of a SDS-polyacrylamide gel (10 % acrylamide) shows the number of bands (top) in the order of their decreasing molecular weight (band 8 = 80 000, band 16 = 54 000 and band 19 = 48 000 daltons) and their relative per cent of total protein (bottom). Protein content was estimated by measuring the area indicated in brackets. Gel is 7 cm long, internal diameter 0.5 cm. Protein, 50 μg, was electrophoresed for 7 h, 10 mA per gel. Phosphate buffer system, pH 7.0, and molecular weight determinations were as previously described [2]. Gels were stained with Coomassie brillant blue.

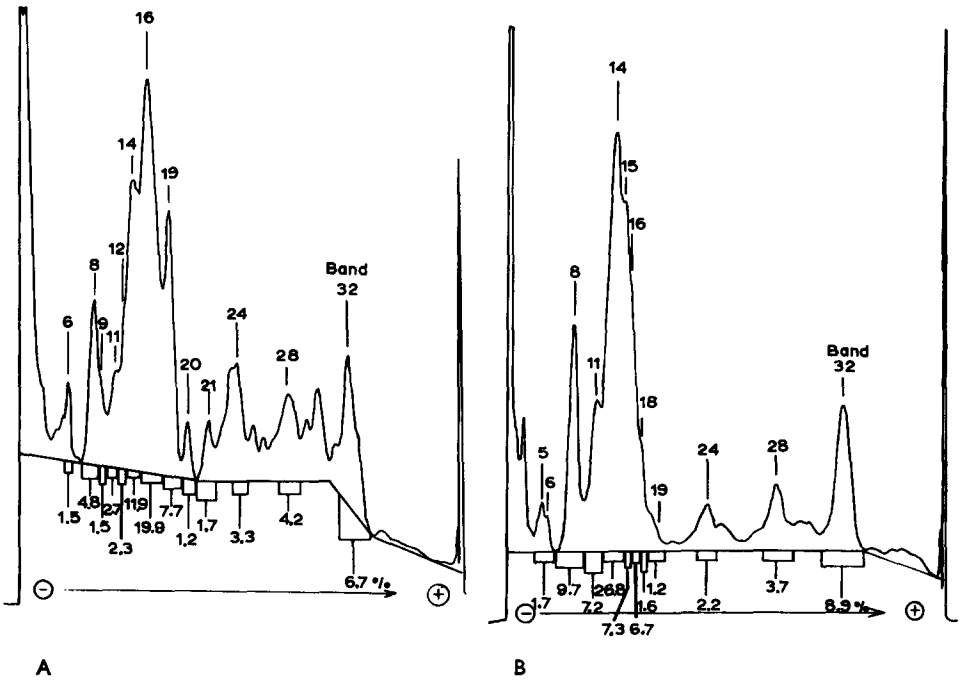

Fig. 2. Resolution and quantitation of all proteins in total liver microsomes (A) and sub-microsomal ammonium sulfate fraction I 40–50 (B) obtained from 3-methylcholanthrene pretreated mice. Running conditions for the above gels were as in Fig. 1 except for time. The run was stopped as soon as the tracking dye, bromphenol blue, reached the end of the gel, about 4 h. Band 32 was identified as cytochrome b_5 using a purified standard which was the generous gift of P. Strittmatter, and has a molecular weight of 17 000.

Fig. 2A demonstrates the resolution of total microsomal proteins obtained when smaller molecular weight microsomal proteins are retained. Fraction I 40–50, Fig. 2B, was the only fraction which had major bands in this lower molecular weight region.

A comparison of scans of ammonium sulfate fractions

Fig. 3 presents scans of all five fractions for oil and phenobarbital pretreated groups. The scans for 3-methylcholanthrene were very similar to oil except for band 16 (see Table I) and are thus not shown. Note that the changes in protein seen in microsomes [2] are sometimes concentrated in one fraction, e.g. band 12 in I 40–50 (Fig. 3B5), and sometimes divided among several fractions, e.g. bands 15 and 16 (Table I). The decrease in band 19 after phenobarbital induction is reflected in I 0–30 (Fig. 3B1), but there also appears to be a decrease in the total amount of this component in sub-microsomal fractions as compared to microsomes. This could be due to selective aggregation of this component, as it is unique in having its single maximum in I 0–30, the least soluble fraction.

Major components of II 30–35

As in our initial studies [1] we were still only able to see three distinct bands, 14, 16 and 18, in our fraction II 30–35 (Fig. 3) despite improved methods. The

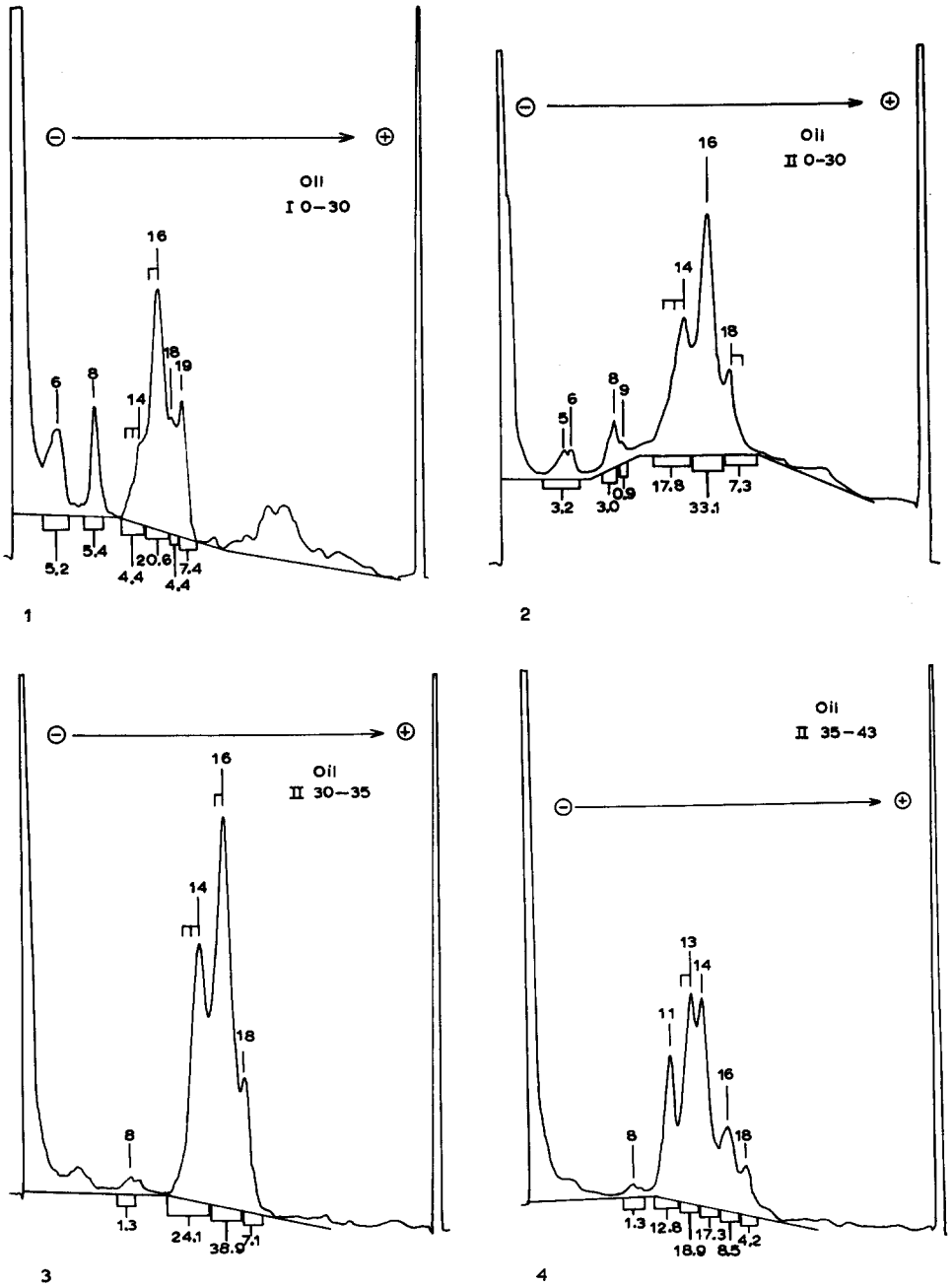

Fig. 3A. See page 679 for legend.

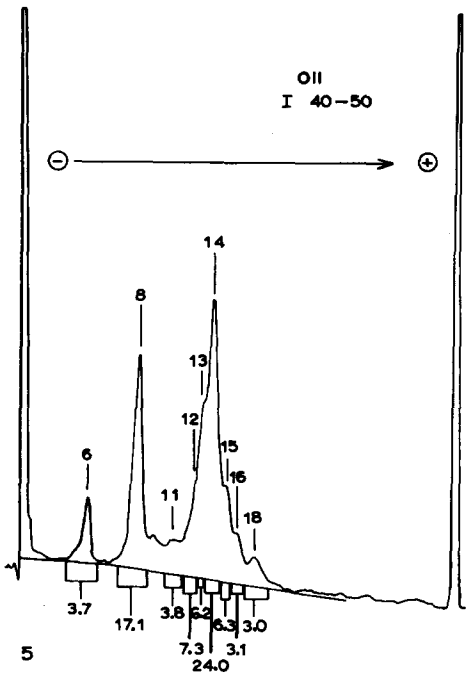

Fig. 3A. See page 679 for legend.

TABLE I

A COMPARISON OF CYTOCHROMES *P*-420 AND *P*-450 TO THE AMOUNT OF BANDS 15 AND 16 IN MICROSOMES AND SUBMICROSOMAL AMMONIUM SULFATE FRACTIONS FROM VARIOUSLY PRETREATED MICE

Values for cytochromes were calculated from CO binding and are given in nmole hemoprotein/mg protein. Values for bands are given in percent relative to total protein. The preparations shown above were made at the same time (180 mice; 60 per pretreatment) in order to keep variations in the preparation to a minimum. Control and phenobarbital data are from the scans in Fig. 3. The values indicated by an asterisk are atypically low indicating the degradation of heme.

	Microsomes	I 0–30	II 0–30	II 30–35	II 35–42	I 40–50
Oil						
Control						
Cytochrome *P*-450	1.0	0.48	1.16	1.4	0.44	0.1
P-420	0.0	0.22	0.34	0.18	0.16	0.1
Band 16	14.4	20.6	33.1	38.9	8.5	3.1
Band 15	5.2	0.0	0.0	0.0	0.0	6.3
Phenobarbital						
Cytochrome *P*-450	2.3	0.32	1.64	1.82*	0.1	0.56
P-420	0.5	0.08	0.50	0.50	0.3	0.36
Band 16	20.0	26.4	31.6	25.3	3.5	6.6
Band 15	14.4	9.1	13.1	6.7	2.3	18.8
3-Methylcholanthrene						
Cytochrome *P*-450	0.68*	1.28	1.72	1.22	1.20	0.68
P-420	0.00	0.36	0.34	0.00	0.12	0.48
Band 16	16.8	27.5	36.8	40.0	23.6	8.0
Band 15	4.8	0.01	0.0	0.0	0.0	11.8

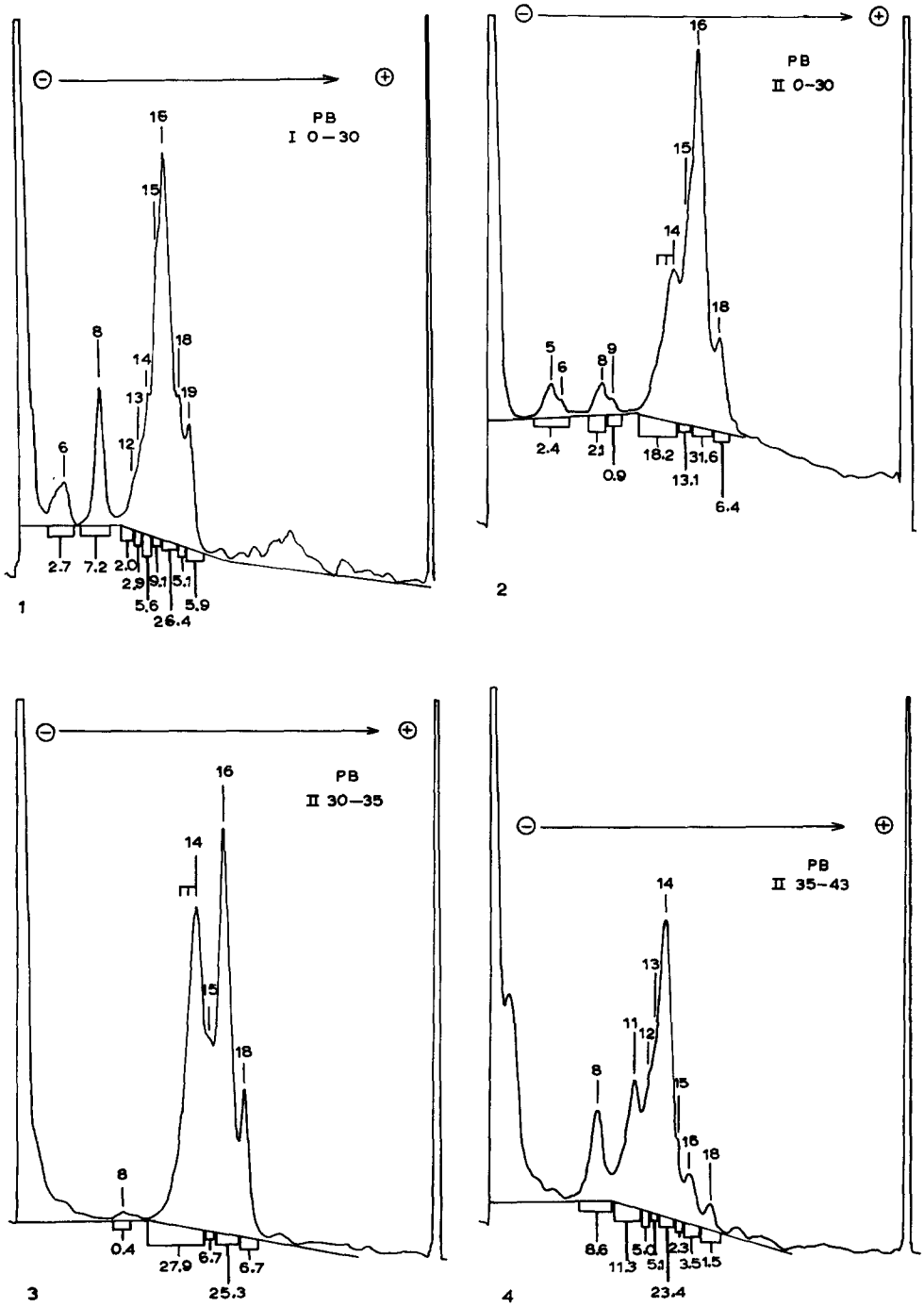

Fig. 3B. See page 679 for legend.

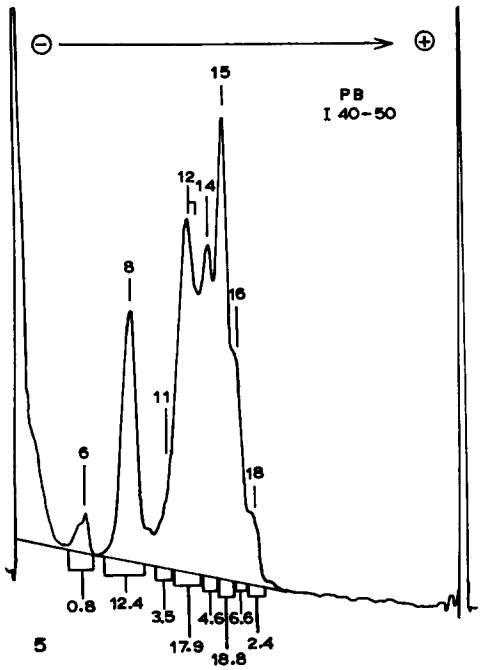

Fig. 3B.

Fig. 3. Resolution and quantitation of major components in sub-microsomal ammonium sulfate fractions from oil control and phenobarbital pretreated mice. The running conditions for these gels were as in Fig. 1.

reported increase in the higher molecular weight component, band 14, after 3-methylcholanthrene induction [20] could not be reproduced in our experiments. This is also supported by our microsomal results [2] which showed no increase in this component. The increase in the lower molecular weight component, band 18, after phenobarbital induction was more difficult to confirm or refute [1, 2]. In these studies the absolute amount of band 18 appeared elevated after phenobarbital treatment, but the relative content was not reproducibly increased. We then tried to come closer to this problem of band 18 induction by using labelling studies carried out as described in Methods.

A comparison of gels stained for protein with their counterparts used in radioactivity measurements (Fig. 4) showed general agreement in optical density and labelling measurements for the same preparation. Band 18, however, does appear to be exceptionally elevated in the case of phenobarbital. Attempts to obtain specific activities, i.e. cpm per mg protein, for labelled bands, however, were hampered by the differences in gel system and resolution of components. These experiments thus tend to confirm the increase in band 18 after phenobarbital pretreatment, but the lack of specific activities and the problem of accounting for the relationship of incorporation time to de novo synthesis and catabolism in vivo made this method only slightly better than direct quantitation of bands by scanning. Further experiments were, therefore, performed by the latter method.

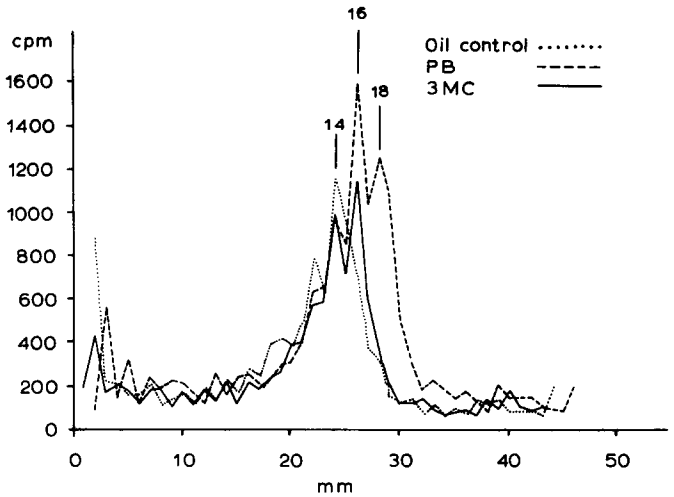

Fig. 4. Counts from slices of preparative gels containing labelled proteins of fraction II 30–35 from oil control and phenobarbital or 3-methylcholanthrene pretreated microsomes. The gel system for preparative gels was the same as described in Fig. 1. The running conditions were 8 mA/gel, 24 h. The longer time was necessary to compensate for the smaller current density in the larger, 1.0 cm internal diameter, gels. The conditions for analytical and preparative gels were so chosen as to yield approximately the same running distance in both systems. The counting efficiency was only 30 per cent due to the quenching effect of the dissolved polyacrylamide. Two preparations, three gels counted per preparation, were made for each group of mice.

Cytochromes P-450, estimated from CO binding, compared with bands 15 and 16

A comparison of CO binding to bands 15 and 16 revealed a relative increase in CO binding after phenobarbital pretreatment which correlated very well with the relative increase in band 15 for total microsomes [2]. However, looking at the absolute values for CO binding and protein increase in microsomes (Table I), one sees that the increase in CO binding is much larger than the increase in band 15 in absolute terms. This means that there should be a change in the binding of CO per mole of protein, i.e. band 15 should have a higher binding coefficient for CO than band 16.

The fact that these two species of cytochrome *P*-450 can be almost completely separated on the basis of their solubility (Table I), which was also to be expected from the differences in amino acid composition and enzymatic properties [21, 22], enabled us to test this hypothesis in our fractions. Much to our surprise, the fraction richest in band 15, I 40–50, showed no disproportionate increase in CO binding. The fraction which did reflect the change in CO binding after phenobarbital pretreatment was II 30–35, the fraction richest in band 16. This fraction derived from 3-methylcholanthrene treated microsomes showed the opposite effect, i.e. band 16 was also elevated but CO binding was decreased. This led to more CO binding, as compared to control, in II 0–30 for 3-methylcholanthrene and more in II 30–35 for phenobarbital groups although the peptide distribution in these fractions appeared similar after induction. A similar change was seen in determining the spectral binding constants, K_s, for metyrapone in fraction II 30–35 and metyrapone's inhibition of *O*-demethylase activity in total microsomes [3]. Thus it appears that band 16, although not induced by phenobarbital [2], is somehow affected to make its heme

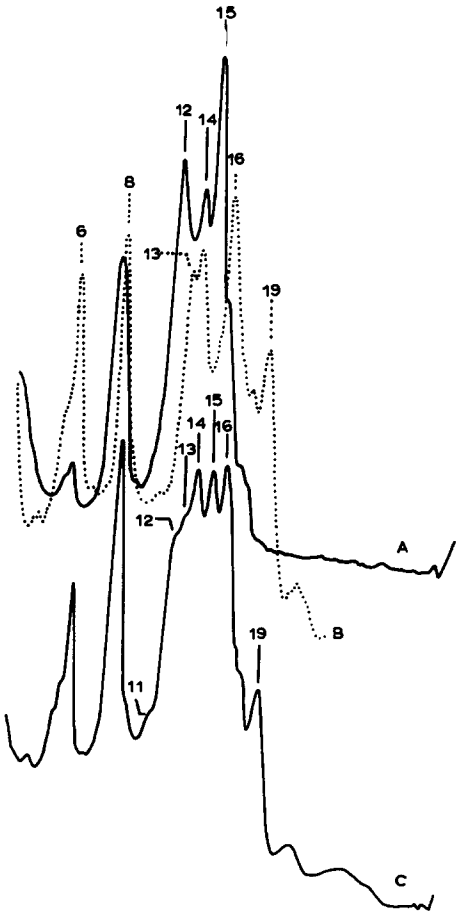

Fig. 5. A comparison of the peptide pattern of corn oil control microsomes with ammonium sulfate fraction I 40–50 from phenobarbital pretreated mice. Running conditions for gels were as in Fig. 1. A = 15 μg protein-I 40–50; B = 50 μg protein-oil control; C = 7.5 μg A plus 25 μg B proteins. Using this mixed-gel method one can easily determine if small differences in running distance for two samples are artifactual or represent two different components, e.g. band 8 vs. bands 15 and 16. The phenobarbital derived fraction is the same as seen in Fig. 3B5.

group more accessible after phenobarbital and less so after 3-methylcholanthrene induction. This could be related to changes in protein composition, e.g. band 18, or a more general effect, i.e. phenobarbital may increase membrane protein solubility or accessibility while 3-methylcholanthrene decreases it.

Hemoprotein content and CO binding

(*A*) *Photometric determination of heme*: Photometric determination of heme (data not presented) also agreed well with the sum of cytochromes *P*-450 and b_5 in our fractions. Thus from the above data, only two proteins could be definitely identified as *P*-450 cytochromes, i.e. bands 15 and 16 showed a direct relationship to CO binding. As related in our labelling studies, changes in the region of band 18

TABLE II

A COMPARISON OF ESR SIGNAL, $g = 2.24$, TO THE AMOUNT OF BANDS 15 PLUS 16 IN AMMONIUM SULFATE FRACTIONS FROM PHENOBARBITAL PRETREATED MICE

	I 0–30	II 0–30	II 30–35	II 35–43	I 40–50
Percent of total protein band 15+16	7.4	24.8	35.4	22.0	6.1
ESR signal amplitude	0.4	0.9	1.2	0.5	0.2

could also be related to other species of *P*-450 proteins, but no concrete evidence for or against such arguments could be found in our fractionation studies. Nevertheless, bands 11, 12, 13 and 14 can be ruled out as possible cytochromes *P*-450 due to their high levels in fractions with little or no CO binding or heme, e.g. II 35–43 (Fig. 3D).

(B) *ESR-studies*: It could, however, be possible that one or more of the components is a hemoprotein of the *P*-450 family, but could not be detected by CO binding in the isolated fractions. In addition to CO binding, another general characteristic of all cytochromes *P*-450 is their unusual heme structure as described by Roeder [23]. This unique heme binding can thus be used to check our CO binding data by semi-quantitative ESR measurements. The low spin triplet, $g = 2.4$, 2.24 and 1.91, which is typical for cytochromes *P*-450 is not as sensitive a parameter as the CO absorption band, but perhaps even more specific. It also has the important advantage of not requiring any manipulation of the protein sample.

The ESR data were in excellent agreement with our CO binding studies (Table II). This would indicate that all the cytochrome *P*-450 heme in our fractions can be measured by CO binding, and that bands 11, 12, 13 and 14 are probably not *P*-450 hemoproteins.

Despite our efforts to determine the number of cytochrome *P*-450 proteins with various methods, heme destruction [24] and non-specific heme binding [25] remain complicating factors in this system. The non-specific binding of heme is of special importance in experiments where heme staining in SDS gels is used as an indicator of cytochromes *P*-450. The electrophoretic conditions for protein to be stained for heme are also different, e.g. a lower SDS concentration, and such gels thus have another peptide pattern when superstained with Coomassie blue [26]. These points, as well as the smearing of bands and fast fading of stain, made this method unsatisfactory for comparison with our gels. Nevertheless, Levin has recently reported on an improved heme staining procedure [27] which could make this method useful in future experiments.

From these results one can say that (1) only bands 15 and 16 appear to correlate with CO binding and ESR data as cytochromes *P*-450; (2) these components are inducible with phenobarbital and 3-methylcholanthrene respectively – the induction with phenobarbital being very strong while that with 3-methylcholanthrene was small and difficult to reproduce [2]; (3) these components have differing solubilities as also reported from isoelectric focusing experiments [21], and appear in totally different complexes upon fractionation; and that (4) band 15 cannot account for all

the changes seen after phenobarbital induction. Here we are probably dealing with a lipid · protein complex and/or protein-protein interactions. These studies thus underscore the necessity of working with complex structures as these hemoprotein effects, as well as those for microsomal flavoproteins, seem to disappear or change in purified systems.

NADH-dependent cytochrome c reductase (EC 1.6.1.1)

In contrast to the studies on hemoproteins, improved resolution of components and functional measurements for the NADH-dependent reductase did not confirm our initial interpretation [1] that band 14 was the NADH-dependent flavoprotein (compare Table III with Fig. 3). As suggested in initial reports, the region between 55 000 and 65 000 molecular weight where band 14 was located was very complex. In addition to band 14, we were later able to demonstrate two additional bands, 12 and 13, with our improved SDS-gel electrophoresis method [2], but this did not improve the agreement between band 14 and the NADH-dependent cytochrome *c* reduction. The improvement of our cytochrome *c* reductase method also indicated that band 14 is not responsible for the NADH-dependent activity. This is also supported by the assignment of a lower molecular weight to this component by Strittmatter [28] as opposed to the earlier studies of Sato [29].

We have found that this NADH-dependent reductase has a maximum activity in fraction I 40–50 (Table III), but is either quantitatively decreased or has a lower specific activity on solubilization as compared to microsomes. The consideration that cytochrome b_5 is always required for NADH-dependent cytochrome *c* reduction [30] was not supported by our results. We obtained as much as 20 per cent of maximal activity in fraction II 30–35 which contained no cytochrome b_5. However, the presence and function of activator and/or supressor proteins in the reaction, as suggested by others [31], is naturally dependent on an exact assignment of some band to this function. From Strittmatter's data for SDS-gels, band 28 (Fig. 2B) would be the most probable candidate for this component. A definitive assignment, however, will

TABLE III

NADH-DEPENDENT CYTOCHROME *c* REDUCTASE IN TOTAL LIVER MICROSOMES AND AMMONIUM SULFATE FRACTIONS FROM VARIOUSLY PRETREATED MICE

Protein concentrations and other conditions were as described in Methods. Preparation 1 is the same as in Fig. 3. The second preparation demonstrates the variation between preparations.

	Microsomes	I 0–30	II 0–30	II 30–35	I 35–43	I 40–50
Control						
Preparation 1	425	20	23	27	9	125
Phenobarbital						
Preparation 1	417	37	17	12	28	140
Preparation 2		27	25	28	12	180
3-Methylcholantrene						
Preparation 1	345	15	17	17	8	150
Preparation 2		12	25	16	12	126

require purification and direct comparison of pure samples and fraction mixtures in SDS-gels.

Higher molecular weight components in the range of 50 000.

The higher molecular weight components resolved by our improved SDS-gel electrophoresis method [2], bands 12* and 13, and that already seen in our initial studies [1], band 11, are especially interesting in so far as they all appear to be glycoproteins [32], and an increase in some component in this region is apparent after phenobarbital induction [2]. The recent isolation of the microsomal UDPglucuronosyl transferase having a molecular weight of 59 000 [33] would indicate that either band 11, 12 or 13 is connected with this activity.

These components demonstrate maxima in fractions II 35–43 and I 40–50 (Fig. 3) as would be expected from the presence of the hydrophilic carbohydrate components. Band 13 was reproducibly most prominent in II 35–43 for oil injected controls. However, this effect was not seen in 3-methylcholanthrene pretreated animals or in total microsomes from oil controls.

Band 13 also appeared elevated in 3 out of 5 phenobarbital preparations for II 35–43, 25 per cent of total protein as compared to 15–18 per cent for oil and 3-methylcholanthrene groups. Band 12 was also increased after phenobarbital pretreatment, especially in fraction I 40–50. Fig. 10 demonstrates that the band seen in this fraction is definitly band 12. This increase also agrees better with microsomal data. We thus propose that only band 12 is induced after phenobarbital pretreatment, and that the apparent increase in band 13 is due to poor resolution of these two components. The fact that bands induced by phenobarbital always appear together in the same fractions from preparation to preparation is also seen as an indication that they are closely associated with one another and that they build a functional, phenobarbital-inducible membrane subunit or compartment.

Band 11 appears to be unchanged by pretreatment of animals and is equally distributed between fractions II 35-43 and I 40-50. These and other glycoproteins in this system will be discussed in detail in a future publication.

NADPH-Dependent cytochrome c reductase (EC 1.6.2.3)

The NADPH-dependent cytochrome *c* reductase of liver microsomes was purified very early [35] and appeared to be easily solubilized. The detergent solubilized form has an approximate molecular weight of 80 000 [36], and is therefore easily identified, even in microsomes, as the only major component in this molecular weight range. This activity is doubled after phenobarbital pretreatment, but this does not

---

* Preliminary experiments have been made, comparing the electrophoretic methods of Laemmli [34] as used by Coon and that of Weber and Osborn as modified in our laboratory. It was found that our band 12 correlates well with Coon's band LM 7, while band 15 must, by definition, be LM 2. There are, therefore, several major differences between these data: (1) cytochrome *P*-450 (LM 2 or band 15) has a higher molecular weight in Coon's experiments; (2) our experiments indicate that at least some of the components in the 50 000 molecular weight range are not *P*-450 hemoproteins; (3) and the number and molecular weight of other components vary for the two methods. Further, band 19 from mouse liver microsomes does not appear to be present in our rat liver microsomes. It is, however, possible that animal origin and species as well as differences in microsomal preparation could contribute to the above differences. A detailed study of this problem is now in progress.

TABLE IV

A COMPARISON OF NADPH-DEPENDENT CYTOCHROME-*c* REDUCTASE AND BAND 8 IN TOTAL LIVER MICROSOMES AND AMMONIUM SULFATE FRACTIONS FROM VARIOUSLY TREATED MICE

Activities are expressed as nmol cytochrome *c* reduced per min and per mg (total) protein or per mg band 8. Protein concentrations and other conditions were as described in Methods. Band 8 is expressed as percent of total protein. The preparations are as in Table III. Note that the activity/mg band 8 is very reproducible for a given fraction despite large variations in the amount of protein from different preparations.

	Micro-somes	I 0–30	II 0–30	II 30–35	II 35–43	I 40–50
Control						
Preparation 1						
Activity/mg Pt	48	14	9	10	8	144
Band 8	10.0	5.4	3.0	1.3	1.3	17.1
Activity/mg Band 8	480	259	333	770	615	843
Phenobarbital						
Preparation 1						
Activity/mg Pt.	105	22	14	5	86	258
Band 8	10.5	7.2	2.1	0.4	8.6	12.4
Activity/mg Band 8	1000	305	666	1040	1000	2080
Preparation 2						
Activity/mg Pt.		30	30	16	28	276
Band 8		9.0	4.6	1.7	2.9	9.0
Activity/mg Band 8		333	652	940	940	3035
3-Methylcho-lanthrene						
Preparation 1						
Activity/mg Pt.	40	12	10	6	8	138
Band 8	10.1	5.2	2.1	1.2	2.6	16.8
Activity/mg Band 8	415	231	476	500	307	822
Preparation 2						
Activity/mg Pt		12	18	12	18	124
Band 8		4.7	6.2	2.5	2.7	12.0
Activity/mg Band 8		256	290	480	668	1032

appear to be due to an increased amount of the flavoprotein, i.e. band 8 [2]. This intriguing result led us to ask which of our fractions reflect this change, and what this could mean.

As seen in Table IV, we found that band 8 demonstrated two maxima, one in I 0–30 and the other in I 40–50, the least and most soluble of our fractions respectively. We also found that phenobarbital pretreatment shifted more of this component into the least soluble fraction I 0–30, while the most soluble fraction, I 40–50, reflected the increase in specific activity. The reductase activity for this fraction was also significantly higher than in microsomes for all treatments. This indicates that there

is something in this fraction which is not only induced by phenobarbital but also enriched for control and 3-methylcholanthrene preparations. The enrichment and induction of bands 12 and 15 appear to fit this pattern.

General comments

With these studies we have been able to demonstrate several interesting effects of phenobarbital and 3-methylcholanthrene:

(1) In the 50 000 molecular weight range we found a decrease in band 19, a very insoluble protein, and an increase in band 18.

(2) We confirmed the assignment of only two protein bands to the CO binding seen in liver microsomes. We also found that these two components differed in their solubility despite similar molecular weights, and appear in different lipid · protein complexes with other microsomal components. It appears, however, that the increase in band 15 after phenobarbital pretreatment could not be solely responsible for the respective increase in CO binding, but rather that the binding of CO and metyrapone to band 16 is also increased in fraction II 30–35 while 3-methylcholanthrene decreases both parameters.

(3) The presence of significant amounts of glycoproteins, bands 11, 12, 13 and 24, and the induction of one of these components, band 12, by phenobarbital is a new aspect in microsomal membrane studies.

(4) The presence of some activating effect for the NADPH-dependent reductase, band 8, in fraction I 40–50 was observed. The appearance of two maxima for this component could also be connected with the biphasic kinetics of the cytochrome *P*-450 reductase; the more soluble and active species perhaps representing the "ordered" component and the insoluble or aggregated form the "unordered" component recently reported by other researchers [37]. The distribution change seen after phenobarbital pretreatment, however, might suggest a more specific role for this compartmentation, which was also seen for cytochrome b_5 [1].

In these effects seen as an entity, the authors find that induction changes in the microsomal membrane appear to be directly connected with the solubility of components, although this may apply only to microsomes. Initial experiments with bovine rod outer segments, however, seem to indicate a more general role for the varying solubilities of membrane proteins.

Further, the most soluble fraction of microsomal proteins contains the maximum for all other known microsomal electron transport activities except for cytochrome P_1-450 [1], i.e. NADPH-dependent (band 8) and NADH-dependent (band 28?) cytochrome *c* reductases, and cytochrome b_5 (band 32), as well as the two major glycoproteins, bands 12 and 24 [32]. These specific differences in the solubility and composition of the cytochrome *P*-450 complexes reflect a similar situation to that seen in mitochondria and thus could justify the classification of the more and less soluble forms to intrinsic and extrinsic positions in the microsomal membrane respectively. Further, this arrangement in the microsomal membrane is most probably related not only to the differing lipid dependences of *P*-450 activities [22, 38, 39], but also indicates that different intermediate steps should be involved in the transfer of electrons from the NADPH-dependent reductase to each of the cytochromes *P*-450, or that two species of the NADPH-dependent reductase exist.

The above data are in partial agreement with the recent report of two highly

purified cytochromes *P*-450 and two additional fractions "contaminated" with various other microsomal components from phenobarbital treated mouse liver [40]. We, however, do not share the opinion of the aforementioned authors that differences in enzymatic activities for the same or similar fractions must be interpreted as an indication of even more *P*-450 cytochromes. It is also possible that the reported "contaminants" actually reflect a complex association of a small number of cytochromes *P*-450 with varying intermediate electron carriers, i.e. NADPH- or NADH-dependent reductases and cytochrome b_5. With such complexes, the liver cell could achieve the inducibility and enzymatic flexibility which mark this system with a minimum of energy.

ACKNOWLEDGEMENT

This work was supported by grant SS 275 A of the Bundesminister für Forschung and Technologie, Bonn, G.F.R.

REFERENCES

1 Mull, R. H., Voigt, T. and Flemming, K. (1975) Biochem. Biophys. Res. Comm. 64, 1098–1106
2 Mull, R. H., Schgaguler, M. and Flemming, K, (1975) Biochem. Biophys. Res. Commun. 67, 849–856
3 Mull, R. H. Hinkelbein, W., Gertz, J. and Flemming, K. (1977) Biochim. Biophys. Acta 481, 407–419
4 Remmer, H. (1959) Naunyn-Schmiedeberg's Arch. exptl. Path. Pharma. 235, 279–287
5 Brown, R. R., Miller I. A. and Miller, E. C. (1954) J. Biol. Chem. 209, 211–222
6 McLennan, D. H., Izagaloff, A. and McConell D. G. (1965) Biochim. Biophys. Acta 131, 59–80
7 Lu, A. Y. H. and Coon, M. J. (1968) J. Biol. Chem. 243, 1331–1332
8 Lu, A. Y. H., Strobel, H. W. and Coon M. J. (1969) Biochem. Biophys. Res. Commun. 36, 545–551
9 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
10 Lu, A. Y. H. and Levin, W. (1972) Biochem. Biophys. Res. Commun. 46, 1334–1339
11 Neville, D. M. and Glossmann, H. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. XXXI, pp. 92–102, Academic Press, New York
12 Ingelman-Sundberg, M. and Gustafsson, J. A. (1977) FEBS Lett. 74, 103–106
13 Maddy, A. H. (1976) J. Theor. Biol. 62, 315–326
14 Matthieu, J. M. and Quarks, R. H. (1973) Anal. Biochem. 55, 317–320
15 Dehlinger, P. I. and Schminke, R. T. (1971) J. Biol. Chem. 246, 2574–2583
16 Passmann, J. M., Radin, N. S. and Cooper, J. A. D. (1956) Anal. Biochem. 28, 484–490
17 Carey, N. H. and Goldstein, A. (1962) Biochim. Biophys. Acta 55, 346–352
18 Schulze, H. U., Gallenkamp, H. and Staudinger, H. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 809–817
19 Appleby, C. A. and Morton, R. K. (1959) Biochem. J. 75, 359–367
20 Alvarez, A. P. and Siekewitz, P. (1973) Biochem. Biophys. Res. Commun. 54, 923–929
21 Dus, K., Carey, D., Goewert, R. and Swanson, R. A. (1976) Hoppe Seyler's Z. Physiol. Chem. 357, 1025–1026
22 Leibmann, K. C. and Estabrook, R. W. (1971) Mol. Pharm. 7, 26–32
23 Roeder, A. and Bayer, E. (1969) Eur. J. Biochem. 11, 89–92
24 Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385
25 McLean A. E. and Garner, R. C. (1974) Biochem. Pharmacol. 23, 475–476
26 Cameron, R., Sharma, R. N., Sweeney, G. D., Farber, E. and Murray, R. K. (1976) Biochem. Biophys. Res. Commun. 71, 1054–1061
27 Levin, W. (1977) Third International Symposium on Microsomes and Drug Oxidations, Berlin, Pergamon Press, in press

28 Spatz, R. and Strittmatter, P. (1973) J. Biol. Chem. 248, 793–799
29 Sato, R., Mihara, K. and Okuda, T. (1973) in Oxidases and Related Redox Systems (King, T. E., Mason, H. S. and Morrison, M., eds.), Vol. 2, pp. 463–467, Univ. Park Press, Baltimore
30 Orrenius, H. U., Ericsson, J. L. E. and Ernster, S. (1965) J. Cell Biol. 25, 627–639
31 Staron, K. and Kaninga, Z. (1974) Acta Biochim. Polon. 21, 55–60
32 Mull, R. H., Schgaguler, M. and Flemming, K. (1975) Biochem. Soc. Trans. 3, 1023–1025
33 Blackburn, G. R., Bornes, M. and Kaspar, C. B. (1976) Biochim. Biophys. Acta 436, 387–398
34 Laemmli, U. K. (1970) Nature 227, 680–685
35 Masters, B. S. S., Williams, C. H. and Kamin, A. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullmann, M. E., eds.), Vol. X, pp. 573–579, Acad. Press, New York
36 Pederson, T. C., Buege, J. A. and Aust, S. D. (1973) J. Biol. Chem. 248, 7134–7142
37 Mataubara, T., Baron, J., Peterson, L. L. and Peterson, J. A. (1976) Arch. Biochem. Biophys. 172, 463–469
38 Lu, A. Y. H., Levin, W. and Kuntzmann, R. (1974) Biochem. Biophys. Res. Commun. 60, 266–273
39 Wang, H., Pfeiffer, D., Kimura, T. and Tchen, T. (1974) Biochem. Biophys. Res. Commun. 57, 93–101
40 Huang, M., West, S. B. and Lu, A. Y. H. (1976) J. Biol. Chem. 251, 4659–4665