CHARACTERIZATION OF MICROSOMAL ELECTRON TRANSPORT COMPONENTS FROM CONTROL, PHENOBARBITAL, AND 3-METHYLCHOLANTHRENE TREATED MICE: I. DISTRIBUTION OF ELECTRON TRANSPORT COMPONENTS IN AMMONIUM-SULFATE FRACTIONS FROM MOUSE LIVER MICROSOMES

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Summary: Using a modified ammonium sulfate fractionation procedure, seven major components were found in various submicrosomal fractions. Four of these proteins could be assigned to known components of the microsomal electron transport system, cytochromes P-450 and b_5, and the NADH- and NADPH-cytochrome c reductases. The similarity of this system to that seen in the separation of mitochondrial enzymes suggests that the lipo-protein complexes of these fractions are particulate in nature and represent functional subunits of the microsomal membrane.

Many researchers have sought changes in protein composition which could be responsible for the differential inducibility of type I and type II substrate activities in the liver microsomal hydroxylase system. It was not until 1965, however, that McLennan (1), seeing the similarity to mitochondria, attempted to separate this system into its subunits, microsomal electron transport particles. Other groups have adopted and refined the methods of McLennan (2,3,4), but at the same time have reverted to examining only the "puriest" fractions and trying to remove all "contaminats".

Seeing a flaw in this logic as discussed again in recent work on mitochondrial systems (5,6), we have returned to the original thesis of McLennan, who proposed that the proteins seen in any given fraction reflect functional and structural relationships within the membrane. We hope that this structural approach to microsomal electron transport may lead to a better understanding of why cytochrome P-450 preparations are "contaminated" with NADH-cytochrome c reductase and vice versa (7,8), and why these particulate preparations all seem to have a similarly large molecular weight (4,8,9).

Material and Methods: Mice of an inbred strain (NMRI/Kißlegg) were received when 7 weeks old. All animals were fed an Altromin R diet and water ad lib. Phenobarbital treated animals were injected twice daily for two days with 70 mg/kg phenobarbital in physiological saline solution and sacrificed by decapitation 48 hours after the last injection. Three methylcholanthrene treated animals were injected once daily for four days with 20 mg/kg 3-methylcholanthrene in 0.1 ml corn oil and sacrificed 24 hours after the last injection. Untreated, saline and corn oil controls were made. All animals were starved 12 hours before sacrificing. Livers were perfused and microsomes sedimented (105,000 g/60 min) from the 25,000 g supernatant. Protein was determined by the method of Lowry (10).

Microsomal electron transport particles were prepared by the method of Lu and Levin (3) with slight modification (Fig. 1). The

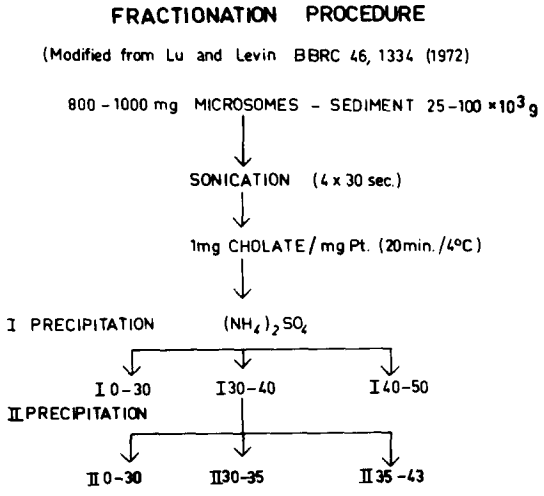

FIGURE 1. $(NH_4)_2SO_4$ fractions are identified by their order of precipitation (I or II) and the range of $(NH_4)_2SO_4$ used for the precipitation in per cent (0-50).

fractions thus obtained were then examined for their content of cytochromes P-450 (11) and b_5 (12) as well as NADH- and NADPH-cytochrome c reductase (13).

An aliquote of each of the five fractions was then extracted by the method of Holtzman (14). The organic phase was collected

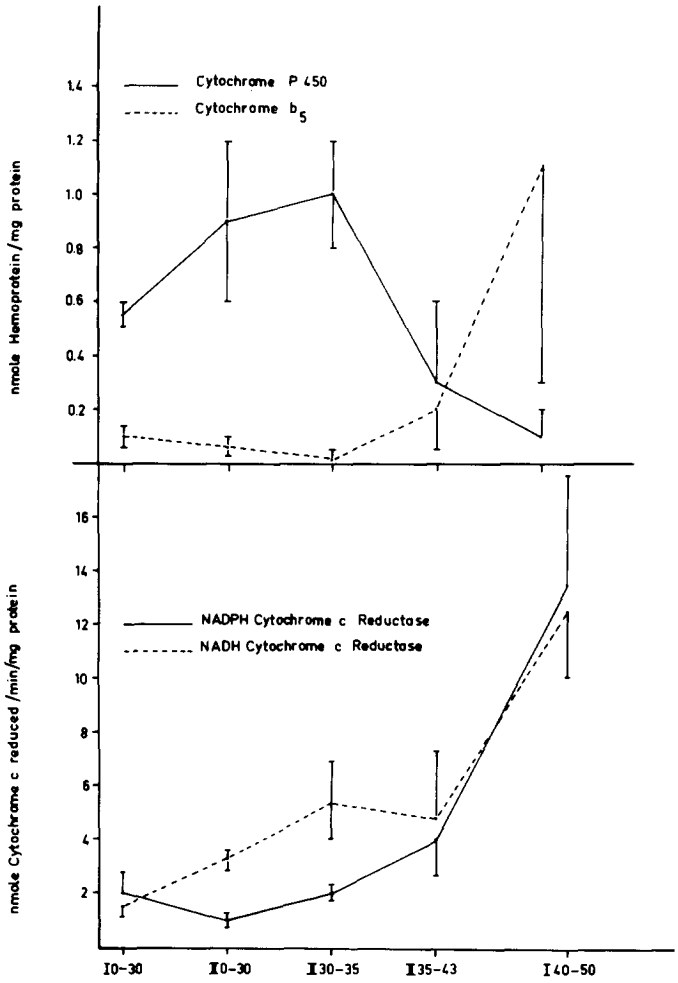

FIGURE 2. Distribution of electron transport components after $(NH_4)_2SO_4$ fractionation of microsomes from 3-methylcholanthrene treated mice.

Particulate fractions are arranged in the order of increasing $(NH_4)_2SO_4$ concentration (x-axis) which in turn reflects the order of increasing solubility. Quantitative measurements of flavin and heme containing proteins (y-axis) were determined as cited in methods. Values are the means and range of 3-4 experiments. Protein concentration was 1 mg/ml for all determinations.

Cytochrome b_5 content in fraction I 40-50 was always higher than in I 0-30, but showed very large variations which appeared more dependent on the preparation than on pretreatment of animals. A detailed study of this problem with purified cytochrome b_5 is in progress.

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for lipid analysis and the protein, which thereby adhered to the glass, was dissolved and subjected to SDS-polyacrylamide[+] gel electrophoresis according to the method of Weber (15) with the

running time being extended to 7 hours for better separation. Molecular weight determinations of the major microsomal peptides by SDS-polyacrylamide[+] electrophoresis was also as previously described (15). The standards were serum albumine = 68,000, catalase = 60,000, ovalbumine = 43,000, and hemoglobin = 15,500. After staining with Comassie blue, the optical density of bands was measured with a Gillford scanning photometer at 610 nm.

Results: From Fig. 2 it is apparent that P-450 is most prominent in fraction II 30-35. This is not in agreement with earlier results of Lu and Levin (3), who reported that the P-450 richest fraction was obtained between 40-50 per cent $(NH_4)_2SO_4$, however, experiments with rat liver microsomes in our lab have failed to confirm these findings. The three other known components of microsomal electron transport NADH- and NADPH-cytochrome c reductase, and cytochrome b_5 are most prominently represented in fraction I 40-50. Preliminary results have shown that both type I and type II hydroxylase activities are present if a mixture of these two fractions, II 30-35 and I 40-50, is used for *in vitro* measurements; however, we feel that useful studies of recombined activity are dependent on a more detailed characterization of all peptides and lipids contained in these fractions. Separation of the proteins on SDS-polyacrylamide gels as shown in figure 3 revealed: (1) seven major components with molecular weights ranging from 10,000 to 100,000 daltons; (2) large quantitative and qualitative differences between ammoniumsulfate fractions within a given preparation; (3) quantitative differences in components for the same ammoniumsulfate fraction from variously treated animals. The largest difference seen in the variously treated groups was an increase in cytochrome P-450 content at elevated ammoniumsulfate concentrations after phenobarbital treatment.

By comparing quantitative functional measurements, seen in figure 2, with band intensity, we have assigned one major peptide to each of the 4 known components (Fig. 3-right). The assignment of activities to electrophoretic components as described in Fig. 4 was supported by a comparison of the ratio of similarly determined components, e.g. reductases or cytochromes, with

[+]SDS = sodium dodecylsulfate

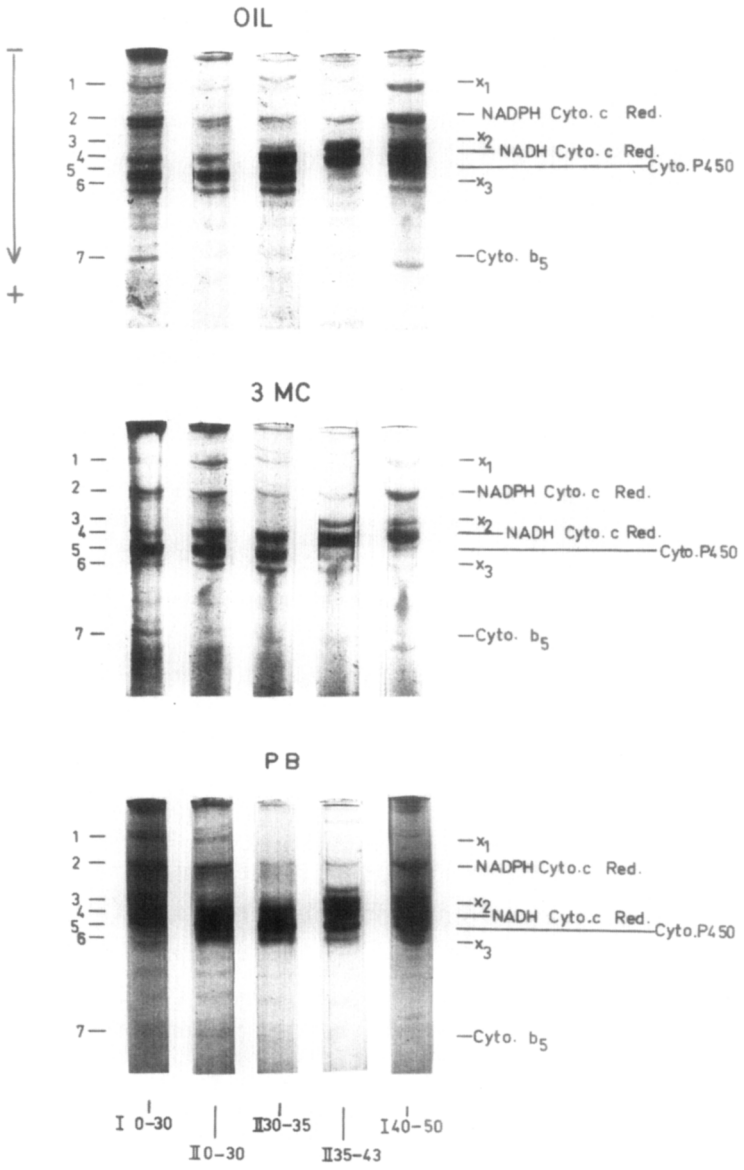

FIGURE 3. SDS-polyacrylamide gels of $(NH_4)_2SO_4$ fractions from variously treated mice.

Gel and buffer system were as cited in methods. Peptides were separated with 8 ma/gel for seven hours. Tracking dye, bromphenol blue, passed through the gel in 3 1/2 hours. Gels were fixed and stained overnight with 0.025 % Comassie brillant blue in a 50 % methanol and 8 % acetic acid solution. Background stain was removed electrophoretically. Major bands were numbered in their order of decreasing molecular weight (left). Each of the flavin or heme containing proteins in Fig. 2 was also assigned to one of these bands (right).

(3MC = three methylcholanthrene; PB = phenobarbital)

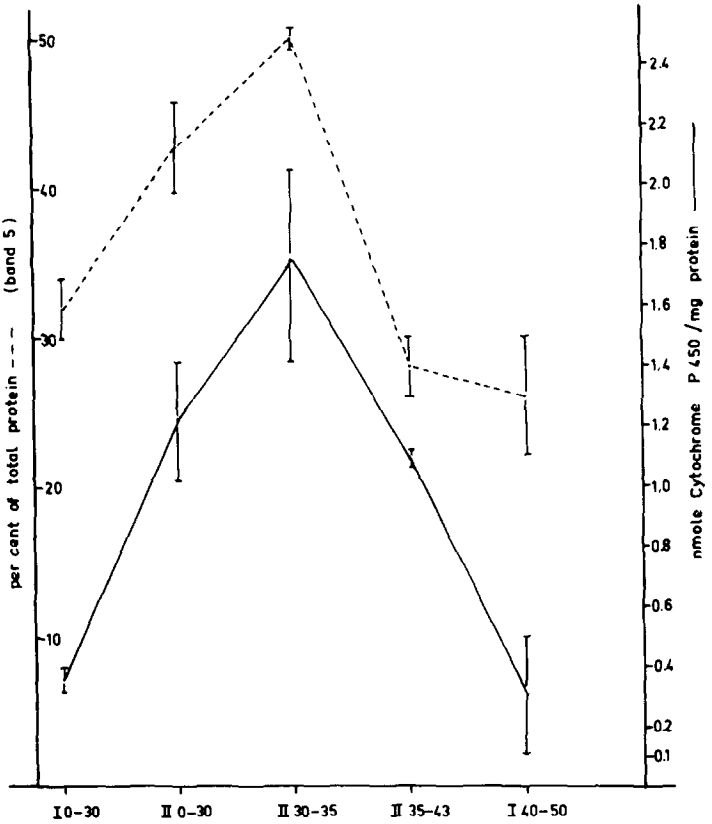

FIGURE 4. Comparision of the distribution curves for band 5 protein and cytochrome P-450 in $(NH_4)_2SO_4$ fractions from phenobarbital treated mice.

Curves are described by increasing $(NH_4)_2SO_4$ concentration on the x-axis as in Fig. 2 and, either per cent of total protein for band 5 derived for the optical density of gel scan (broken line-left), or cytochrome P-450 content derived from CO binding spectra (solid line-right) on the y-axis. As seen in this example from a phenobarbital preparation, band 5 distribution always gave the best fit with respect to cytochrome P-450 content and was thus the most likely candidate for the cytochrome P-450 peptide. Assignment of the other three components of microsomal electron transport was accomplished by the same method.

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the ratio of optical density for the respective bands (Table 1).

The known molecular weights for the four components of microsomal electron transport, cytochromes b_5 (16), and P-450 (3,4), and the NADH (8) and NADPH (17) dependent cytochrome c reductases, fit their migration pattern in the SDS-polyacrylamide gels and therefore lend further support to our assignment of the bands to their respective functional activities. Proteins of lower molecular weight, less than 30,000, were no longer linear with res-

TABLE 1. A comparison of the ratios of NADPH- to NADH-cytochrome c reductase activities with those of band 4 to band 5 in $(NH_4)_2SO_4$ fractions from 3-methylcholanthrene treated mice.+

	Cytochrome c Reductase (nmole cyto.c reduced min/mg/protein)			Band (% of total protein from o.d.)		
Fraction	NADPH	NADH	Ratio	2	4	Ratio
I 0-30	1.95	1.45	1 : 0.70	11.0	7.0	1 : 0.63
II 0-30	1.20	3.20	1 : 2.70	9.0	16.0	1 : 1.80
II 30-35	2.40	5.70	1 : 2.40	6.0	14.0	1 : 2.32
II 35-43	3.80	4.20	1 : 1.10	7.0	22.0	1 : 3.10*
I 40-50	13.30	12.70	1 : 0.95	19.0	20.0	1 : 1.05

+Band 4 protein in fraction II 35-43 appears elevated due to the presence of another peptide of similar molecular weight in this fraction.(A detailed study is in progress.)

pect to molecular weight in this system, but band 7's position would approximate the lower molecular weight of cytochrome b_5.

Discussion: In these experiments, we have been able to separate the membrane bound components of microsomal electron transport into five fractions. On the basis of previous studies (1) also supported by recent work on mitochondrial and microsomal membranes, we suggest that the composition of these particles reflects the specificity of a non-random protein distribution within the membrane as suggested by others (18,19). This contention is supported by the recent experiments of Welton and Aust who used iodination and trypsin digestion in combination with SDS-polyacrylamide gel electrophoresis for structural analyses of total microsomes. They could thus demonstrate a trypsin and H_2O_2 resistant membrane fraction with a peptide pattern similar to that of our fraction II 30-35 (20).

The banding pattern of our fraction II 30-35 agreed well with

that of Alvarez's P-450 preparation as well as with his observation that only one band increased parallel with P-450 content (4). The latter stands in direct contradiction to many current interpretations of this system (3,21,22,23). Band 6 was also regularly increased in fraction II 30-35 after phenobarbital induction. Only our band 4 data were in contradiction with Alvarez as this peptide was only irregularly increased after 3-methylcholanthrene treatment, and similar results were observed in corn oil treated animals. On the other hand, our results are in agreement with those of Sato (8) which assign a peptide of ca. 62,000 molecular weight to the NADH-cytochrome c reductase activity of microsomes.

The most interesting concept which can be drawn from these data is that multitudinous functional activities found in microsomes probably are more dependent on a varied association of the same components than on the presence of various species of a given protein. This concept not only places a smaller demand on protein synthesizing systems, but also suggests that the major role of electron transport membranes is compartmentalization. For example, assuming the above is correct, the two types of cytochrome b_5 recently described by Archakov (24) could be the same protein appearing in different membrane subunits. Exactly this behaviour is reflected in the precipitation of cytochrome b_5 in the dissimilar fractions I 0-30 and I 40-50 (Fig. 2 and 3).

The task of proving the specificity of these protein associations and defining a mechanism which directs them will be difficult. We hope, that by extending these studies to whole microsomes and applying better analytical methods, progress in this direction can be made.

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