CHARACTERIZATION OF MICROSOMAL ELECTRON TRANSPORT COMPONENTS FROM CONTROL, PHENOBARBITAL AND 3-METHYLCHOLANTHRENE TREATED MICE: II. RESOLUTION AND QUANTITATION OF CYTOCHROMES P-450 AND P_1-450 AND THE SO-CALLED "FACTOR X" IN SDS-POLYACRYLAMIDE GELS OF TOTAL MICROSOMES

Robert H. Mull, Margaret Schgaguler and Kurt Flemming

Institute of Biophysics and Radiobiology, University of Freiburg, Albertstraße 23, 78 Freiburg, West Germany

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Summary: By improving the resolution of microsomal proteins in SDS-polyacrylamide gels, we have succeeded in assigning one major protein not only to each of the four known components of microsomal electron transport but also to the proposed components, cytochrome P_1-450 and "Factor X". The increases in P-450 protein after PB induction and P_1-450 protein after 3-MC induction were comparable to the respective increases in CO binding. They demonstrated molecular weights of 56,000 and 54,000 daltons respectively. The "Factor X" protein, molecular weight = 48,000, appears to be of non-heme iron character, and probably is an intermediated electron carrier between flavo- and hemoproteins.

Introduction: Intensive research on liver microsomes over the past few years has revealed a complicated system of electron transport in this membrane fraction. This system appears to be divided into two branches; an NADPH dependent flavoprotein (1, 2,3) coupled with cytochrome P-450 (4,5) and an NADH dependent flavoprotein (6,7) coupled with cytochrome b_5 (8,9). These four known components appear to be linked with one another (10,11), perhaps by way of an often proposed but undefined "Factor X" (3,12). A second cytochrome P-450 protein specifically induced by 3-MC, the so-called P_1-450 or P-448, has also been proposed (13,14,15), but has eluded researchers of this system until now. The assignment of each of the four known components to one major protein in SDS-polyacrylamide gels from sub-microsomal fractions (16) has led us to undertake a detailed examination of these components in the microsomal membrane, where the proteins are in their "native" state and enzymatic functions are retained.

3-MC = three methylcholanthrene; PB = phenobarbital
NHI = non-heme iron

Methods: Treatment of animals, functional assays, and other techniques not mentioned here were as previously described (16). The O-demethylation of para-nitroanisol and acetanilid hydroxylation were determined by the methods of Netter (17) and Leibman (18) respectively.

The SDS-polyacrylamide gel electrophoresis method was based on that of Weber and Osborn (19). Amperage, however, was increased from 8 to 10 mA per gel. Gels were fixed, stained with Comassie blue, and destained by the method of Fairbanks (20). Quantitation of the bands was as described by Matthieu (21). All chemicals were of biochemical or analytical grades and commercially available. Purified cytochrome b_5 was the generous gift of P. Strittmatter.

Results and Discussion: A comparison of 8 mA with 10 mA gels

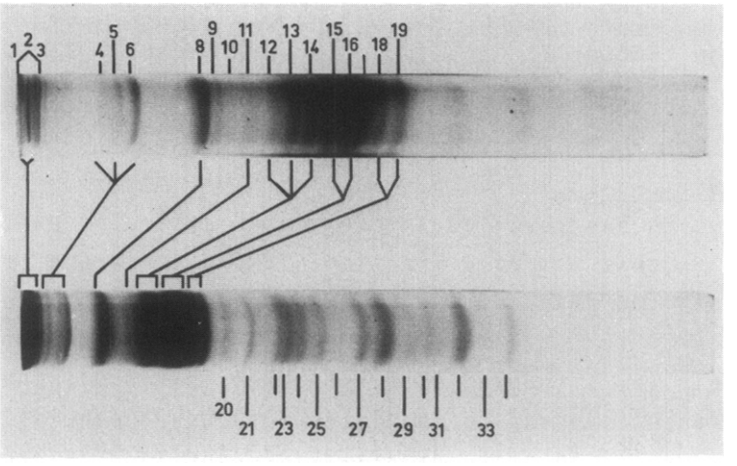

FIGURE 1. Resolution of total microsomal proteins in SDS-polyacrylamide gels.
Gels were loaded with 50 µg of microsomal proteins from PB-treated mice and stained and destained as described in Methods. Upper gel was run with 10 ma/gel for seven hours; lower gel with 8 ma/gel until tracking dye migrated into lower buffer as described by Welton and Aust (approximately 4 hours). Gels were 5 mm internal diameter and 7 cm long. The improved resolution of major component around 50,000 daltons was accomplished at the cost of losing cytochrome b_5 and other low molecular weight components.
Cytochrome b_5, band 32, was identified with the help of a 90% pure standard. Assigments of other components was as previously described.

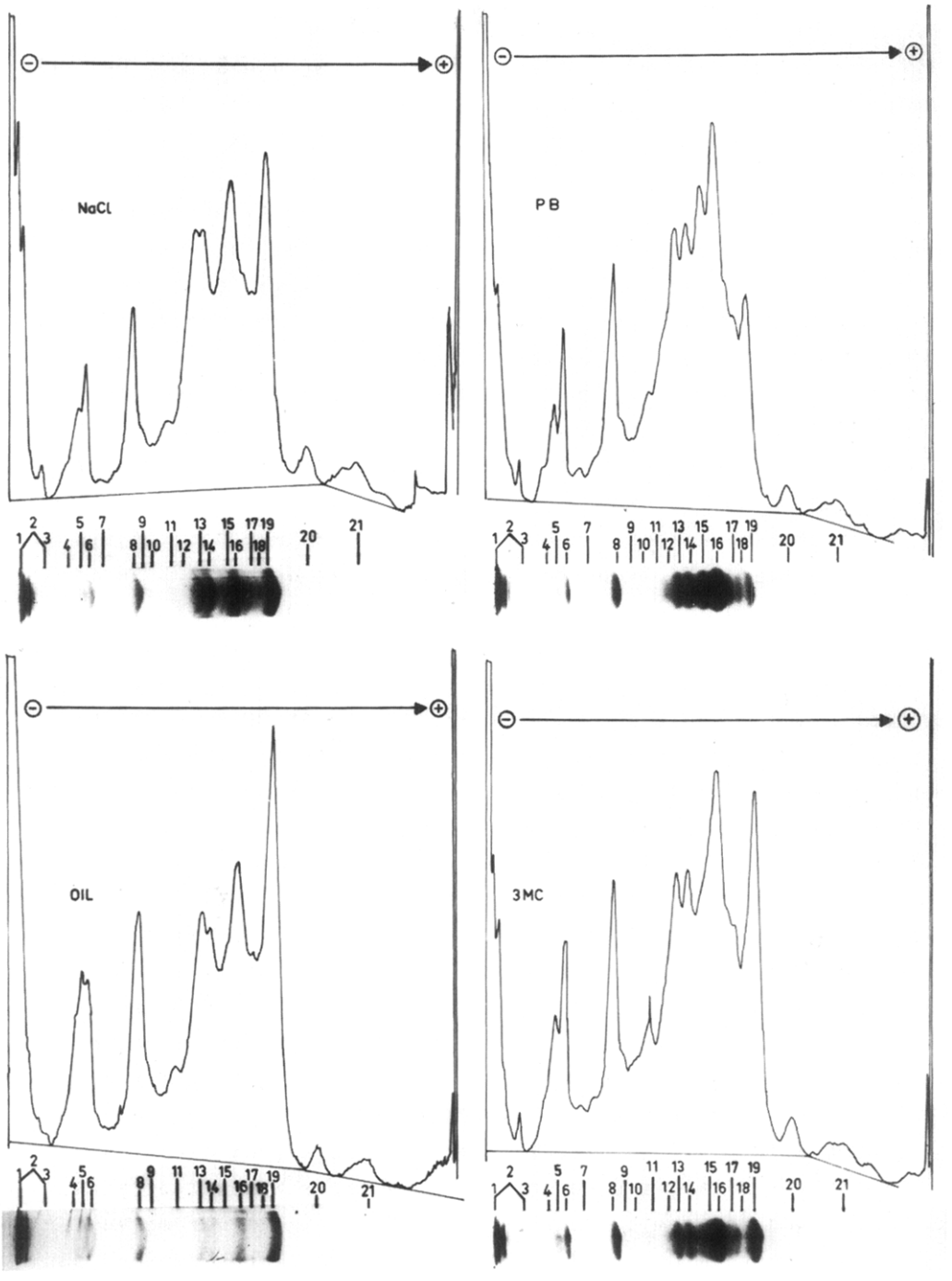

FIGURE 2. A comparison of microsomal proteins from variously treated mice.

Bands were assigned to functional activities as previously described: 8-NADPH-cytochrome c reductase, 11-Unknown 1, 12 + 13-Unknown 2, 14-NADH-cytochrome c reductase, 15-cytochrome P 450, 16-cytochrome P_1 450, 19-factor x.

(Fig. 1) demonstrates the improved resolution of the major microsomal components with molecular weights around 50,000 daltons. Using PB and 3-MC induction, quantitation of electrophoretic components, and parallel enzymatic assays, we then examined the distribution of microsomal electron transport components in total microsomes. From Fig. 2 it is apparent that no significant variations are seen between saline or oil injected controls. Oil and 3-MC treated groups were the problem children of these studies showing the largest fluctuations in electrophoretic and enzymatic experiments. The resolution of proteins in polyacrylamide gels after 3-MC induction was impaired by an inexplicable but recurring diffuse appearence or "smearing" of the bands. This in turn made any minor protein changes insignificant for this group of microsomes.

An increase was seen in band 16, molecular weight = 54,000 daltons, after 3-MC induction while band 15 was less than or equal to controls. After PB induction the opposite effect was seen; an increase at 56,000 daltons, band 15, while band 16 remained unchanged. A large decrease in band 19, 48,000 daltons, was also seen in microsomes from PB induced animals. A closer comparison of gels revealed an apparent decrease in band 11 and/or increase in 12 or 13; however, the major changes in bands 15, 16 and 19 appear to be responsible for the major differences in activity between control, PB and 3-MC induced microsomes.

The above is not in agreement with the most recent work of Welton and Aust in rat liver microsomes (22). These researchers found only increases in microsomal proteins which were comparable to changes in partially purified cytochrome P-450 preparations from rats, while we found increases and decreases in microsomal proteins which varied considerably from partially purified cytochrome P-450 preparation from mice. We have found the partially purified preparations from rats (23) and mice (16) to be similar, and our preliminary experiments indicate that rat and mouse microsomal proteins are of similar molecular weights except for band 19. Thus the contradictions between our experiments and those of Welton and Aust do not appear to be derived from species differences, but rather from differing assignments of the nine proteins, around 50,000 daltons in microsomes to the four seen in purified cytochrome P-450 preparations. A detailed study of this problem is in progress.

A summary of concomitant band quantitation and microsomal activity measurements is presented in Tables I and II. A comparison of PB and 3-MC induced changes in CO binding to band 15 and 16 strongly support the assignment of P-450 to the former and P_1-450 to the latter band. The ratio of 1:4 for P-450: P_1-450, however, does not agree with the predicted value of 1:1 for control animals (15, 24). These data seem to confirm the long

TABLE I

PER CENT OF CONTROL		BANDS		PER CENT OF TOTAL MICROSOMAL PROTEIN				
PB : NaCl	3MC : OIL			NaCl	PB	OIL	3MC	UN-TREATED
117 ± 26	73 ± 32	8 NADPH cyto.c Red.	1		7.9	8.3	6.2	13.6
			2	10.0 7.2	9.4 10.5	10.0	4.1 10.1 10.7	9.8
			3	8.3	9.3	8.5	8.9	8.3
				8.5±1.4	9.3±1.1	8.9±0.9	8.0±2.8	10.6±2.7
122 ± 42	69±14	11	1		2.9	4.1	3.3	3.5
			2	3.3 2.9	3.5 4.9	3.6	1.9 4.6 4.8	4.9
			3	3.7	3.2	4.0	2.9	3.7
				3.3±0.4	3.6±0.9	3.9±0.3	3.5±1.2	4.0±0.8
121±13	103± 3	12 + 13	1		14.5	11.9	12.6	14.5
			2	13.2 12.8	17.4 15.7	10.3	10.4 12.6 13.7	12.5
			3	11.1	11.8	10.0	10.4	11.2
				12.4±1.1	14.9±2.4	10.7±1.0	11.9±1.5	12.7±1.7
89±17	120±25	14 NADH cyto.c Red.	1		6.5	9.4	10.4	11.9
			2	10.0 9.0	7.4 9.7	6.3	9.3 9.5 9.4	7.8
			3	8.6	7.3	8.3	8.4	11.1
				9.2±0.7	7.7±1.3	8.0±1.6	9.4±0.7	10.3±2.2
286±11	39±34	15 P_{450}	1		16.6	6.9	3.9	7.4
			2	4.1 4.1	12.2 11.7	5.2	0.0 3.9 5.1	3.2
			3	4.9	13.5	4.2	2.5	5.1
				4.4±0.5	13.5±2.2	5.4±1.4	3.1±2.0	5.2±2.1
109±0.8	160± 37	16 P_1 450	1		15.7	13.8	20.8	16.1
			2	16.6 15.3	17.7 16.6	14.0	28.0 17.1 15.9	14.0
			3	18.0	19.7	14.8	20.6	19.0
				16.6±1.4	17.4±1.7	14.2±0.5	20.5±4.7	16.4±2.5
48± 4	97±34	19 factor x	1		7.0	11.2	12.9	15.4
			2	17.3 17.1	7.6 8.9	17.9	21.1 12.3 16.7	17.2
			3	9.3	4.5	14.6	8.5	14.2
				14.5±4.6	7.0±1.8	14.5±3.4	14.3±4.8	15.6±1.5

TABLE I. Per cent of total microsomal proteins for major bands in SDS-polyacrylamide gels.

Per cent of total proteins and the mean ± S.D. (right) are shown for 3 microsomal preparations (vertical rows, 1,2 or 3). e.g. groups by mice variously pretreated, sacrified and prepared at the same time. Per cent of respective controls (left) was calculated for parallel experiments (horizontal rows) from the same preparation. A variation analysis according to Fisher revealed significant differences ($P<0.001$) between control and induced animals. Significant changes in individual bands are best seen as per cent of control ± S.D.

TABLE II

PER CENT OF CONTROL		COM-PONENTS		FUNCTIONAL ACTIVITY MEASUREMENTS				
PB : NaCl	3MC : OIL			NaCl	PB	OIL	3MC	UN-TREATED
272 ± 98	193 ± 92	CYTOCHROME	P_{450}	1 0.41 2 0.71 3 0.96 0.69 ± 0.28	0.91 1.48 3.70 2.03 ± 1.50	0.29 0.72 0.91 0.64 ± 0.32	0.86 1.16 1.10 1.04 ± 0.16	0.76 1.10 0.93 ± 0.24
115 ± 18	163 ± 20		b_5	1 0.28 2 0.23 3 0.28 0.26 ± 0.03	0.37 0.22 0.33 0.31 ± 0.08	0.26 0.22 0.21 0.23 ± 0.03	0.38 0.40 0.33 0.37 ± 0.04	0.25 0.25 0.25 ± 0.0
198 ± 36	112 ± 17	CYTO C RED	NADPH	1 18 2 44 3 30 31 ± 13	32 78 72 61 ± 25	17 50 31 33 ± 17	22 48 34 35 ± 13	39 28 34 ± 8
79 ± 7	87 ± 9		NADH	1 452 2 397 3 379 409 ± 38	361 281 321 321 ± 40	397 471 320 396 ± 76	351 366 305 341 ± 32	628 363 496 ± 187
188 ± 17	115 ± 21	OXYGEN ASSAY	Type I	1 2 2.5 3 3.9 2.7 ± 0.3	4.4 7.8 6.1 ± 2.4	3.2 3.5 3.4 ± 0.2	3.2 4.5 3.9 ± 0.9	2.6 3.8 3.2 ± 0.8
149 ± 2	166 ± 48		Type II	1 2 1.5 3 1.0 1.3 ± 0.4	2.2 1.5 1.9 ± 0.5	1.9 1.2 1.6 ± 0.5	2.5 2.4 2.5 ± 0.1	1.7 1.1 1.4 ± 0.4

standing theory that two different CO binding hemoproteins are present in liver microsomes and can be differentially induced by PB or 3-MC pretreatment in vivo.

The decrease in band 19 after PB induction and its equimolar ratio to the known components of microsomal electron transport in control microsomes make this protein the best candidate for "Factor X". The decrease in PB treated animals also suggest a connection between this protein and a non-heme iron component found by Montgomery (25) and ourselves (26) using two different methods. The NHI character of this component is further supported by the recent description of such a protein in liver microsomes by Strittmatter (27) which has a similarly large molecular weight.

On the one hand, the decrease in band 19 appears parallel with a decrease in NADH-cytochrome c reductase activity. The fact that Strittmatter's NHI protein was also connected with NADH dependent electron transport would fit these data, suggesting that these components are one and the same. Further support of this hypothesis is found in the work of Weber et al. on probable path-

ways for cytochrome c reduction in microsomes (28).

On the other hand, the increase in NADPH-cytochrome c reductase activity after PB induction does not appear to be derived from a change in the quantity or ratio of the respective flavoproteins, band 8 and 14, but rather on the change in some other component as already suggested by Staron (29).

Our data, indicating that the relative content of the NADPH dependent flavoprotein, band 8 remains unchanged after PB induction although the NADPH dependent cytochrome c reduction is greatly increased, contrasts with present views on this component (29,30,31). Thus it appears probable that band 19 and/or band 15 represent the key to regulation and cooperation between the two microsomal electron transport chains.

The induction of more O-demethylation, type I, by PB and more acetanilid-hydroxylation, type II, by 3-MC was typical for this system, but no simple relationship of P-450 to type I or P_1-450 to type II activity could be seen. This system thus appears to be much more complex than originally postulated. In this respect, such small but significant changes as those seen in bands 12 and 13 after PB induction must be more closely studied. It is important to note that these components, which represent more than ten per cent of the total microsomal protein, are most probably glycoproteins (32). These studies thus promise to open a new road to understanding the dependence of membrane structure on glycoproteins and their role in microsomal electron transport.

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