A NOVEL ELECTROPHORETIC PATTERN OF INDUCTION OF RAT LIVER MICROSOMAL MEMBRANE POLYPEPTIDES BY 2-ACETYLAMINOFLUORENE, NITROSAMINE AND AZO DYE ADMINISTRATION

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SUMMARY

The electrophoretic patterns of the polypeptides of the microsomal membrane fraction of the livers of rats treated with various agents were compared. Administration of phenobarbital, or of benzo[a]pyrene or 3-methylcholanthrene, resulted in specific increases of membrane polypeptides corresponding to cytochrome P-450 and cytochrome P-448 species respectively. Administration of 2-acetylaminofluorene, diethylnitrosamine, dimethylnitrosamine, N,N-dimethyl-4-aminoazobenzene or 3'-methyl-N,N-dimethyl-4-aminoazobenzene resulted in a marked increase of 2 other polypeptides, migrating just ahead of the phenobarbital-responsive cytochrome P-450 species. Preliminary evidence suggests that at least one of these 2 polypeptides may contain heme. The results indicate that administration of these N-containing carcinogens to rats results in a common electrophoretic pattern of induction of 2 specific microsomal membrane polypeptides. This pattern is different from those observed with classical inducers of the rat liver mixed-function oxidase system.

INTRODUCTION

Induction of different mixed-function oxidase species in liver by administration of PB[1] and MC is a well-established fact (1). Recent purification studies have elegantly confirmed the multiplicity of forms of cytochrome P-450 and related hemoprotein species in liver microsomes (2, 3). A number of workers have documented this concept by comparing the electrophoretic patterns of liver microsomal membrane polypeptides from animals treated with PB, MC or other inducers using SDS-polyacrylamide electrophoresis (4-6). Conversion of the carcinogens 2-AAF, DAB, 3'me-DAB, DEN or DMN to reactive metabolites requires microsomal participation (7, 8). However, no electrophoretic studies on the patterns of microsomal membrane polypeptides from the livers of rats treated with these agents appear to have been reported. In this study we have compared

[1]Abbreviations: PB, phenobarbital; MC, 3-methylcholanthrene; BP, benzo[a]pyrene; 2-AAF, 2-acetylaminofluorene; DAB, N,N-dimethyl-4-aminoazobenzene; 3'me-DAB, 3'-methyl-N,N-dimethyl-4-aminoazobenzene; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; i.p., intraperitoneal; RER and SER, rough and smooth endoplasmic reticulum; AHH, aryl hydrocarbon hydroxylase; EIB, ethyl isocyanide-binding; MW, molecular weight; CO, carbon monoxide.

the electrophoretic patterns of rat liver microsomal membrane polypeptides after the administration of PB, MC, BP, 2-AAF, DAB, 3'me-DAB, DEN and DMN. The results show that the 5 latter compounds increased substantially the amounts of 2 microsomal membrane polypeptides whose electrophoretic migrations differ from those of the polypeptides induced by PB, MC or BP administration. Whether the polypeptides induced by the N-containing carcinogens are cytochrome P-450 species or other microsomal constituents involved in xenobiotic metabolism remains to be firmly established; some support for the former view has been obtained.

MATERIALS AND METHODS

For studies of induction by 2-AAF, male Fischer rats weighing 120-130 g were generally fed a basal diet or a 0.02% or a 0.05% 2-AAF diet (9) and sacrificed 1, 2, 4, 7 and 14 days subsequently. Treatments with other inducers were as follows: (i) PB - one dose of 80 mg/kg in 0.9% NaCl i.p. per day for 3 days (ii) MC - one dose of 40 mg/kg in corn oil i.p. (iii) BP - one dose of 40 mg/kg in corn oil i.p. (iv) DAB or 3'-me-DAB - one dose of 300 mg/kg in corn oil by stomach tube (v) DEN - one dose of either 50 or 200 mg/kg i.p. (vi) DMN - one dose of either 6 or 25 mg/kg i.p. The rats given DAB, 3'-me-DAB, DEN and DMN were sacrificed 4 days after the dose of carcinogen. PB-, MC- and BP-treated rats were sacrificed 24 h after the last injection. Animals were fasted overnight and total microsomal fractions (or in some cases separated RER and SER fractions) were prepared as described previously (10). The microsomal fractions were "stripped" of ribosomes (10) prior to electrophoresis. Approximately 50 µg aliquots of microsomal protein were subjected to flat-plate polyacrylamide-SDS electrophoresis using a 10% acrylamide separating gel and a 5% acrylamide-stacking gel (cf. 11). The tracking dye (bromphenol blue) was allowed to migrate 18 cm from the origin at which time (6 h) electrophoresis was terminated and the gels stained with Coomassie blue. For cytochrome P-450 measurements, aliquots of liver were homogenized in a mixture of 0.05M tris-HCl (pH 7.5)-0.1M KCl, the microsomal fractions prepared by differential centrifugation and re-suspended in 0.1M sodium phosphate (pH 7.4). AHH activity was measured (12) on similarly prepared tissue, except that the 9000 g supernatant was used. Cytochrome P-450 was measured (13) using a Hitachi Perkin Elmer 356 dual wavelength spectrophotometer operating in the difference mode. The extinction coefficient $Em_{450-490}$ = 91 cm^{-1} for conventional difference spectroscopy was used (13). EIB spectra were examined at pH 7.4 similarly, following addition of 1 µl ethyl isocyanide to the microsomes in the sample cuvette (14).

RESULTS

Illustrative of the electrophoretic findings made in the present study is Fig. 1, a photograph of a flat plate electrophoretic separation of microsomal membrane preparations from variously treated rats. The polypeptide patterns of 2 control liver preparations are shown in slots 7 and 14. The zones in the area of the gel of principal interest (i.e. in the MW area of approximately 45-55,000, corresponding to the MW range of the rat and mouse liver microsomal membrane polypeptides previously shown to be induced by PB (cytochrome P-450) and MC (cytochrome P-448) (cf. refs. 4-6)) are numbered a-k in the right hand margin of slot 14. The marker polypeptides used are shown in slot 8. The alteration of microsomal membrane polypeptide pattern produced by PB is shown

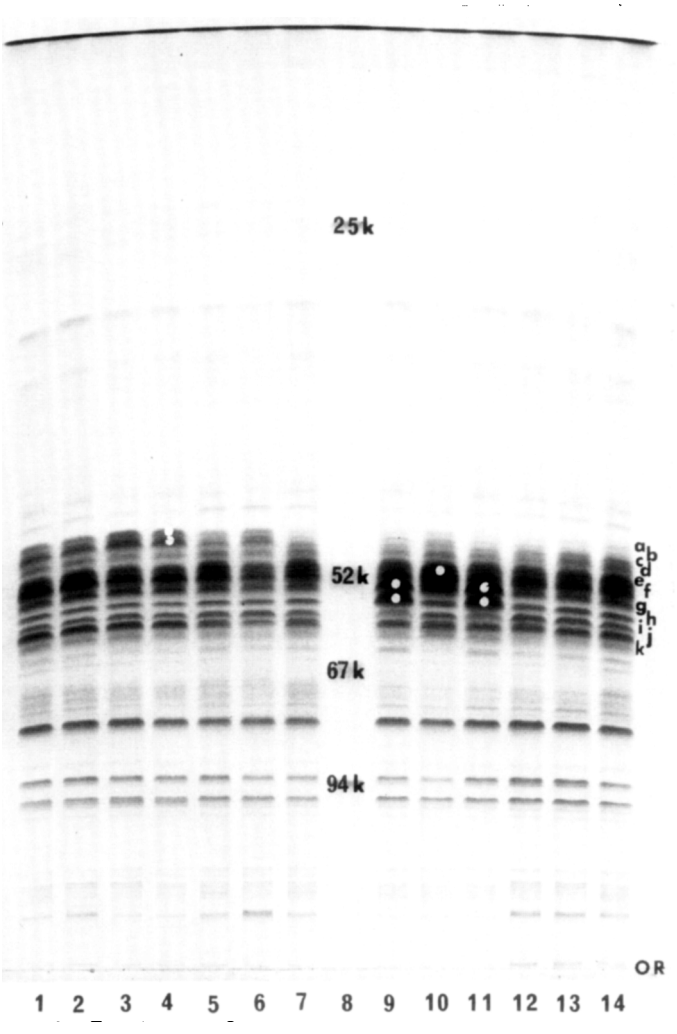

Fig. 1: Flat-plate polyacrylamide-SDS gel electrophoresis of the microsomal membrane polypeptides from variously treated rats.

The microsomal membrane fractions were "stripped" of ribosomes prior to electrophoresis. Approximately 50 µg of microsomal protein was applied to each slot. Electrophoresis was performed at room temperature for 6 h. The gel was stained with Coomassie blue. The samples applied were:
Slots 1 and 2, Membranes from 2 rats fed a 0.02% 2-AAF diet for 2 weeks
Slots 3 and 4, Membranes from 2 rats fed a 0.05% 2-AAF diet for 2 weeks
Slots 5 and 6, Membranes from 2 rats injected with 50 and 200 mg/kg of DEN respectively and sacrificed 4 days later
Slots 7 and 14, Membranes from 2 control rats
Slot 8, Marker proteins (from top to bottom: 25 K = chymotrypsinogen A; 52 K = heavy chain of gamma globulin; 67 K = rat serum albumin; 94 K = rabbit muscle phosphorylase a)
Slot 9, Membranes from 1 rat injected with MC (see text)
Slot 10, Membranes from 1 rat injected with PB (see text)
Slot 11, Membranes from 1 rat injected with BP (see text)
Slots 12 and 13, Membranes from 2 rats subjected to partial hepatectomy and sacrificed 48 and 24 h later respectively.

TABLE I. SUMMARY OF EFFECTS OF ADMINISTRATION OF VARIOUS AGENTS ON THE INDUCTION OF RAT MICROSOMAL MEMBRANE POLYPEPTIDES a AND b

TREATMENT	RESPONSE	TREATMENT	RESPONSE
0.02% 2-AAF,		DAB	++++
14 days	+++	3'me-DAB	++++
0.05% 2-AAF,		DEN	++
1 day	0	DMN	++
2 days	+++	PB	0
4 days	++++	MC	0
7 days	++++	BP	0
14 days	++++	CONTROL	0

Induction refers to an increase of intensity of the polypeptides labelled a and b in Fig. 1. The observed increases of the intensities of polypeptides a and b are scored 0 to ++++, as evaluated by visual examination of stained gels. 0 is the intensity of polypeptides a and b in preparations from control animals. As specific examples, the increases of intensity of polypeptides a and b shown in channels 1 and 2 (Fig. 1) would be scored as +++, those in channels 3 and 4 as ++++ and those in channels 5 and 6 as ++. At least 2 animals were subjected to each type of treatment. The dosage schedules of DAB, 3'me-DAB, DEN, DMN, PB, MC and BP were as described in the Materials and Methods. The animals given 2-AAF were started on diets containing 2-AAF at zero time, maintained on the diet for the times indicated, and sacrificed after fasting overnight.

in slot 10; the principal effect is a noticeable intensification of zone e. The alterations produced by MC and BP administration are illustrated in slots 9 and 11; 2 zones (g and h) are seen to be markedly intensified in each case. In general, the above results on the effects of PB and MC administration are in good agreement with those previously reported (4). The novel findings made in the present study are shown in slots 1-6. Slots 1 and 2 show the patterns of microsomal membrane polypeptides from the livers of 2 rats fed a 0.02% 2-AAF diet for 2 weeks; slots 3 and 4 show similar preparations from 2 rats fed the 0.05% 2-AAF diet for 2 weeks. The most conspicuous changes in all these 4 samples are a marked intensification of zones a and b; none of the zones increased by PB or BP or MC is seen to be noticeably increased in intensity. Microsomal membrane samples from 2 rats injected with DEN and sacrificed 4 days later are shown in slots 5 and 6 respectively. Again, zones a and b are seen to be notably increased in intensity with little changes in intensity of other neighbouring zones being evident. Slots 12 and 13 show microsomal preparations from 2 rats subjected to partial hepatectomy (ref. 15) 48 and 24 h previously; little alteration from the patterns of control liver (slots 7 and 14) is evident. We next investigated the temporal aspects of induction of these polypeptides by 2-AAF administration and also the ability of several other compounds to produce

TABLE 2. SOME PROPERTIES OF THE MICROSOMES OF CONTROL AND VARIOUS DRUG-TREATED RATS

TREATMENT	CYTOCHROME P-450* n moles/mg prot.	AHH ACTIVITY units/mg prot.	ETHYL ISOCYANIDE SPECTRUM 454:430 Ratio (pH 7.4)
Control	0.74 ± 0.04	119 ± 13	0.54 ± .01
2-AAF	1.03 ± 0.07	213 ± 22	0.60 ± .08
PB	2.18 ± 0.16	206 ± 27	0.85 ± .01
MC	1.24 ± 0.11	1038 ± 128	1.67 ± .07

All values represent the means and standard deviations of results obtained from analyses on microsomes of 3 rats. Control rats were fed basal diet (9). Induction was with: (a) 4 days of the 0.05% 2-AAF diet (b) 3 daily injections of PB (100 mg/kg) and (c) one injection of MC (40 mg/kg). Cytochrome P-450 and AHH values were significantly increased in all the treated groups compared to the control groups at $p<0.01$. One unit of AHH activity is the amount of enzyme catalysing the formation per min. at 37°C of hydroxylated product causing fluorescence equivalent to that of 1 p mole of 3-hydroxybenzo(a)pyrene.
*The absorption maximum for the reduced CO-treated microsomal hemoprotein complex was approximately 448 nm for MC-treated microsomes and 450 nm for the other preparations.

a similar effect. The results of these and other related studies are summarized in Table 1. A key consideration that arose from the above results was to determine whether the 2 polypeptides whose amounts were increased by administration of 2-AAF or the other agents listed in Table 1 represented components of mixed-function oxidases and/or some other microsomal components involved in xenobiotic metabolism. We have confined our attention to induction by 2-AAF and have approached this problem in 2 ways: (i) estimation of total cytochrome P-450 activity and comparison of some of its parameters in liver samples from MC-, PB-, and 2 AAF-treated rats (ii) attempts to determine whether the 2 inducible polypeptides contain heme, using mild conditions of electrophoresis that permit retention of some heme in association with polypeptide chains of cytochrome P-450 species and thus allow their specific detection by staining with benzidine (5). Table 2 summarizes data on the total cytochrome P-450 contents, AHH activities and EIB spectral studies. 2-AAF administration resulted in: (a) a moderate increase (40%) of total cytochrome P-450 activity over control values (b) a moderate increase of AHH activity, comparable to that produced by PB but much less than that produced by MC (c) an EIB spectrum essentially similar to that of control liver and quite different from that produced by MC administration. Electrophoresis and staining with benzidine of liver microsomal samples from control, PB- and 2-AAF- treated animals revealed a prominent benzidine-positive zone in all 3 types of samples corresponding in migration to

that of that PB-responsive cytochrome P-450 polypeptide species. In the 2-AAF-treated samples, a fainter benzidine-positive zone was observed just ahead of this zone. Subsequent staining with Coomassie blue revealed that this latter zone corresponded approximately in migration with the slower-migrating of the 2 polypeptides induced by 2-AAF. In our experience the resolution in the 45-55,000 MW region of these gels was inferior to that obtained with the regular gels and both the quantitative and qualitative distribution of the resolved polypeptides differed from that obtained with the regular gels. In particular, only 1 polypeptide was observed to be present and to be increased in amount following 2-AAF administration in the area where 2 zones (a and b, Fig. 1) were resolved in regular gels.

DISCUSSION

These studies have demonstrated a response ("induction" in the broad sense referred to by Conney (16)) of rat liver microsomal polypeptides to administration of various carcinogens (2-AAF, DAB, 3'me-DAB, DEN and DMN) that differs from the responses produced by classical inducers (e.g. PB and MC) of mixed-function oxidases. This novel response occurs within 72 h after commencing to feed 2-AAF. Other studies have revealed that both "stripped" RER and SER membrane fractions show approximately equal concentrations of the 2 polypeptides; this suggests that it is unlikely that the increase of the 2 specific polypeptides observed in the total microsomal fraction is due to contamination of this fraction by polypeptides from other organelles whose subcellular distribution has been altered by processes of cell injury produced by the administered carcinogens. The most important question raised by the present observations is whether the 2 induced polypeptides constitute components of a specific sub-class of mixed-function oxidase (cytochrome P-450 type species) or whether they are other microsomal constituents involved in drug metabolism. The studies reported here do not answer this question unequivocally. The microsomes of 2-AAF-treated rats showed a moderate (40%) increase of cytochrome P-450, a normal absorption maximum for the CO derivative of reduced microsomal hemoprotein of 450 nm (as distinct from 448 nm for the MC-induced P-448 species (cf. 17)) and an EIB spectrum similar to that of control microsomes. What appears clear is that if hemoprotein induction is involved, it does not seem to be of the cytochrome P-448 type. Moreover, studies to date suggest that appearance of these 2 polypeptides is not accompanied by any major spectral shift from that exhibited by normal microsomes. The experiments using benzidine reagent to detect hemoproteins do support the conclusion that at least one of the 2 polypeptides is a hemoprotein species. Our present working hypothesis is that the response observed here represents at least in part an adaptive response of a

sub-class of cytochrome P-450, perhaps involved in N-hydroxylation. The following support this hypothesis: (1) N-hydroxylation is involved in the metabolism of 2-AAF and possibly certain of the azo dyes (7, 18, 19) (2) Hemoproteins migrating just ahead of the PB-responsive cytochrome P-450 species and corresponding approximately in migration to the 2 polypeptides described here have been described in rabbit liver (e.g. LM_1 species of Haugen et al. (ref. 3)) (3) Malejka-Giganti et al. (20) have reported that administration of 2-AAF to Sprague-Dawley rats stimulates microsomal N-hydroxylation apparently involving a mixed-function oxidase not of the MC-induced variety, an observation with which our spectral studies are consistent. No electrophoretic data or studies with other inducers were reported by these workers. It is also apparent, however, that the adaptive response described here may involve other microsomal constituents involved in drug metabolism e.g. flavoprotein oxidase (21), glucuronyl- and sulpho- transferases etc. (7). The reported MW values for cytochrome b_5 (22) and NADPH cytochrome P-450 reductase (23) of 16,700 and 80,000 respectively would appear to make them unlikely candidates. Further studies are in progress to distinguish between these alternatives. One final point also merits comment. Studies still in progress have revealed that rabbits, guinea pigs, hamsters or mice (Swiss strain) fed a 0.05% 2-AAF diet for 4 days failed to show the marked increase in the 2 polypeptides reported here for the rat. Of particular interest in this regard are previous observations that in rabbits, guinea pigs, hamsters and some strains of mice, 2-AAF is much less carcinogenic to the liver than in the rat (18). The effects described here thus apparently provide another example of the marked variation among species of cell constituents involved in drug metabolism in general (24) and of 2-AAF metabolism in particular (25).

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