Multiplicity of Induction Patterns of Rat Liver Microsomal Mono-Oxygenases and Other Polypeptides Produced by Administration of Various Xenobiotics

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Induction of hepatic microsomal mono-oxygenase species after administration of various xenobiotics is a well-documented phenomenon. To examine the number and specific species of rat liver microsomal membrane polypeptides involved in such responses, we have used sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to analyse microsomal fractions from animals treated with a number of important xenobiotics. The following are the principal points to have emerged from this study. 1. A minimum of twelve electrophoretically distinct patterns of induction of haemopolypeptides and other polypeptides could be distinguished after administration, either singly or in certain combinations, of phenobarbital, 3-methylcholanthrene, polychlorinated biphenyls, 2-acetylaminofluorene, safrole (or isosafrole), pregnenolone-16 α -carbonitrile and ethanol. The patterns consisted of various permutations of the amounts of eight polypeptides of 47000–56000 mol.wt., of which at least three were haemopolypeptides. The possible identities of these polypeptides, which included species of cytochrome *P*-450, cytochrome *P*-448 and epoxide hydratase, are discussed. 2. Agents (3-methylcholanthrene, benzo[*a*]pyrene, polychlorinated biphenyls, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and β -naphthoflavone) that result in the induction of cytochrome *P*-448 caused a marked increase in two polypeptides of 54000 and 56000 mol.wt., whereas safrole and isosafrole induced only the former polypeptide. 3. Administration of 2-acetylaminofluorene resulted in the induction of two polypeptides; evidence is presented that suggests that one of these is a species of epoxide hydratase [cf. Levin, Lu, Thomas, Ryan, Kizer & Griffin (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3240–3243] and that the other may be a novel haemopolypeptide. 4. The overall results emphasize the complexity of the responses exhibited by rat liver microsomal fractions to the administration of xenobiotics.

Administration of agents such as phenobarbital and 3-methylcholanthrene results in induction [used here in the broad sense of Conney (1967) to describe an increase in the amount of one or more polypeptides, irrespective of the mechanism involved] of microsomal mono-oxygenases involved in the metabolism of numerous drugs, steroids, fatty acids, chemical carcinogens and other compounds (cf. Conney, 1967; Schulte-Hermann, 1974; Weisburger, 1978). SDS/polyacrylamide-gel electrophoresis has proved to be a useful technique for monitoring the responses of specific liver microsomal membrane polypeptides to the administration of xenobiotics. (The term polypeptide is used here to describe components resolved by SDS/polyacrylamide-gel electrophoresis, as it generally cannot be excluded that the conditions of SDS/polyacrylamide-gel electrophoresis may have dissociated native proteins into polypeptide subunits.)

Abbreviation used: SDS, sodium dodecyl sulphate.

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Alvares & Siekevitz (1973) compared the effects of administration of phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls on the electrophoretic pattern of rat liver microsomal membrane polypeptides, and were able to show that the biphenyls induced both cytochromes *P*-450 and *P*-448. Haugen *et al.* (1976) demonstrated the potential of the same approach for analysing the responses of mouse liver microsomal membrane polypeptides to various xenobiotics, including pregnenolone-16 α -carbonitrile. We have shown (Cameron *et al.*, 1976a) that administration of 2-acetylaminofluorene (and of certain other hepatocarcinogens) to rats induces two liver microsomal membrane polypeptides; the novel pattern of induction produced by 2-acetylaminofluorene was clearly different from those resulting from administration of phenobarbital and 3-methylcholanthrene. Ohnishi & Lieber (1977) have demonstrated that chronic administration of ethanol to rats results in the induction of one or two specific liver

microsomal membrane polypeptides. Dickens *et al.* (1978) have shown that a novel hepatic microsomal haemopolypeptide was induced by the administration of isosafrole to rats.

In the present work we have attempted, using an SDS/polyacrylamide-gel electrophoretic system that resolves 55–60 polypeptides from rat liver endoplasmic-reticulum membranes (Sharma *et al.*, 1978a), to provide at least partial answers to the following questions. (1) How many polypeptides can be shown to be involved in the induction responses of rat liver microsomal membranes to xenobiotics? As inducers, we selected the agents mentioned above [namely phenobarbital, 3-methylcholanthrene, polychlorinated biphenyls, 2-acetylaminofluorene, ethanol, isosafrole (and safrole) and pregnenolone-16 α -carbonitrile]. Closely related to this question is the identification of the functions of the polypeptides involved in these responses, and, in particular, of the two polypeptides induced by administration of 2-acetylaminofluorene. (2) How many electrophoretically distinct patterns of induction can be generated, using the above agents singly (in some cases comparing short- and longer-term administration of the same compounds), or in certain combinations? The results of this study clearly indicate the existence of a greater degree of complexity in the responses of rat liver microsomal fractions of xenobiotics than has previously been demonstrated by SDS/polyacrylamide-gel electrophoresis. Specifically, we have shown that a minimum of eight polypeptides is affected by the administration of the agents studied and that a minimum of twelve different patterns of induction involving these polypeptides can be recognized. In addition, the observations assist in clarifying the nature of the polypeptide responses of rat liver microsomal membranes to agents that induce cytochrome *P*-448 and also to 2-acetylaminofluorene. Preliminary accounts of certain aspects of this work have been presented (Sharma *et al.*, 1978b; Murray *et al.*, 1979).

Materials and Methods

Adult male Fischer rats (approx. 150 g body wt.) were used for all experiments except those involving chronic feeding of ethanol, for which adult female Sprague–Dawley rats of similar body weight were employed. For studies of induction by 2-acetylaminofluorene, animals were fed on a diet containing 0.05% 2-acetylaminofluorene (Cameron *et al.*, 1976a) and killed 6 days later. Treatment with other chemicals was as follows: phenobarbital, one dose of 80 mg/kg in 0.9% NaCl intraperitoneally per day for either 2 or 3 days (short-term) or 5 days (longer-term); 3-methylcholanthrene, one dose of 40 mg/kg in corn oil intraperitoneally (short-term) or three or five daily injections of the same dose (longer-term); poly-

chlorinated biphenyls (Araclor 1254; Analabs, North Haven, CT, U.S.A.), one dose of 25 mg/kg in corn oil every third day for a total of five injections; safrole (Aldrich Corp., Milwaukee, WI, U.S.A.), one dose of 100 mg/kg in corn oil intraperitoneally per day for 3 days; isosafrole (ICN Pharmaceuticals, Plainsview, NY, U.S.A.), one dose of 150 mg/kg in corn oil intraperitoneally for 3 days; pregnenolone-16 α -carbonitrile (Searle Laboratories, Chicago, IL, U.S.A.), one dose of 50 mg/kg in corn oil intraperitoneally per day for 3 days; 2,3,7,6-tetrachlorodibenzo-*p*-dioxin (Dow Chemicals, Midland, MI, U.S.A.), one dose of 5 μ g/kg intraperitoneally dissolved in 1,4-dioxan (250 μ g/ml), the rats being killed 5 days later; β -naphthoflavone, one dose of 80 mg/kg in corn oil intraperitoneally, the rats being killed 48 h later. Control animals were also injected with the solvents mentioned above that were used to dissolve the various agents administered. In a number of experiments, animals were maintained on the diet containing 0.05% 2-acetylaminofluorene for 6 days and given injections of phenobarbital, 3-methylcholanthrene or phenobarbital + 3-methylcholanthrene on days 5, 6 and 7. Animals given 2-acetylaminofluorene + polychlorinated biphenyls were fed on the 0.05% 2-acetylaminofluorene-containing diet continuously, during which time the polychlorinated biphenyls were given in the dosage schedule described above. The control and ethanol (36% of total calories) liquid diets for the Sprague–Dawley rats were the same as described previously (Villeneuve *et al.*, 1976); the animals were fed on these diets for 21 days. All animals were starved overnight and killed by decapitation approx. 16 h after the last injection of each chemical, except where otherwise indicated above. At least four animals were used for each treatment described.

Preparation of liver microsomal membrane fractions and electrophoresis by the system of Laemmli (1970) (on a 10% polyacrylamide separating gel) were performed as described previously (Cameron *et al.*, 1976a; Sharma *et al.*, 1978a). The following molecular-weight standards were used (approx. mol.wts. given in parentheses): horse cytochrome *c* (11700), sperm-whale myoglobin (17200), bovine pancreas chymotrypsinogen A (25000), chicken egg ovalbumin (45000), bovine serum albumin (67000) (all from Schwartz/Mann, Orangeburg, NY, U.S.A.), the heavy chain of human IgG (52000) (kindly donated by Dr. K. J. Dorrington, Department of Biochemistry, University of Toronto) and rabbit muscle phosphorylase *a* (94000) (from Sigma Chemical Co., St. Louis, MO, U.S.A.). For detection of haemopolypeptides, the conditions of electrophoresis [with the buffer system of Fairbanks *et al.* (1971)] and staining by tetramethylbenzidine were those of Thomas *et al.* (1976). A preparation of epoxide hydratase (EC 4.2.1.63) was purified from control rat

liver essentially by the method of Knowles & Burchell (1977) (cf. Levin *et al.*, 1978). Data on the cytochrome P-450 content, ethyl isocyanide spectra and aryl hydrocarbon hydroxylase activities of control, phenobarbital-, 3-methylcholanthrene- and 2-acetylaminofluorene-treated rats (Fischer strain) have been presented previously (Cameron *et al.*, 1976a), as have similar data characterizing the properties of microsomal fractions from control and chronic ethanol-treated rats (Sprague-Dawley) (Villeneuve *et al.*, 1976).

Results

Typical electrophoretic results for most of the xenobiotics studied here are illustrated in Fig. 1. Most of the responses involved eight polypeptides within the narrow range of 47000–56000 mol.wt.; these are assigned the numerals I–VIII in order of increasing molecular weight in both the description of this and certain subsequent Figures. In general, the patterns of polypeptides exhibited by the test samples (slots 2–9 and 11) differed (with respect to the amount of one or more of polypeptides I–VIII) from the pattern of the control samples (slots 1 and 10) and also from each other. An exception to this second statement is provided by safrole (slot 7) and isosafrole (slot 8) treatments, which resulted in essentially similar patterns. Further descriptions of the differences in polypeptide patterns shown will be presented when Fig. 3 is described. However, two further points merit comment now. Firstly, essentially identical electrophoretic results were observed when at least three other specimens of each type of sample were analysed. Secondly, three other polypeptides in addition to polypeptides I–VIII were noted to be altered in amounts after certain of the treatments; these are indicated by white dots in Fig. 1. That indicated in slot 3 was consistently increased in amount after administration of phenobarbital (five daily injections), polychlorinated biphenyls and pregnenolone-16 α -carbonitrile, whereas that indicated in slot 6 was consistently decreased in amount after administration of 3-methylcholanthrene (five daily doses). The polypeptide indicated in slots 7 and 8 was consistently increased in amount after administration of safrole or isosafrole.

The electrophoretic pattern resulting from chronic administration of ethanol is shown in Fig. 2(a). The pattern obtained with the microsomal fraction from the animal fed on ethanol chronically (slot 3) is seen to be different from those obtained with the control microsomal fractions (slots 1 and 2) and from the patterns produced by the agents shown in Fig. 1. The observed difference is that ethanol treatment produced a notable increase in the amount of polypeptide VI; this increase was observed in each of six test samples analysed.

The results presented in Fig. 1 demonstrated that treatment with 2-acetylaminofluorene (slot 4) induced both polypeptides I and III. We reasoned that it should be possible to generate additional novel patterns of induction of microsomal membrane polypeptides by first pretreating animals with 2-acetylaminofluorene (to induce polypeptides I and III) and then injecting certain of the other agents (i.e. phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls), whose patterns of induction had been previously established (cf. Fig. 1). Results from such a study are given in Fig. 2(b). The various treatments (slots 8–11) resulted in patterns of induction that generally reflected both the treatment with 2-acetylaminofluorene (cf. slot 6) and the respective effects on the other polypeptides that resulted when phenobarbital, 3-methylcholanthrene, phenobarbital+3-methylcholanthrene and polychlorinated biphenyls (slots 2–5 respectively) were administered without 2-acetylaminofluorene. Essentially identical electrophoretic results were observed when at least three other specimens of each type of sample were analysed.

The various results illustrated in Figs. 1 and 2 are summarized in schematic form in Fig. 3. Only effects on polypeptides I–VIII are shown. Each pattern represents the composite results obtained from analyses of the microsomal fractions from at least four individual animals. The principal results depicted will be presented and clarified with regard to certain points by briefly summarizing the observed responses of the eight individual polypeptides to the various agents studied. Only responses that are considered moderate or marked will be considered; certain relatively minor effects on the amounts of the individual polypeptides that are shown in Fig. 3 will not be commented upon, although they were consistently observed. (1) Polypeptide I was markedly increased in amount by treatment with 2-acetylaminofluorene and moderately increased in amount by treatment with phenobarbital, polychlorinated biphenyls and chronic ethanol. (2) Polypeptide II was markedly elevated in amount after administration of 3-methylcholanthrene for 3 days or more, moderately elevated after administration of phenobarbital for 2 days or more and moderately to markedly elevated after treatment with polychlorinated biphenyls. (3) Polypeptide III exhibited a profile of responses to the xenobiotics that was similar to polypeptide I. (4) Polypeptide IV was moderately induced by all of the treatments, except 3-methylcholanthrene. (5) Polypeptide V was moderately to markedly induced after treatment with phenobarbital, polychlorinated biphenyls, safrole (and isosafrole), pregnenolone-16 α -carbonitrile and ethanol. A consistent feature of the response to pregnenolone-16 α -carbonitrile was that the front edge of polypeptide V migrated somewhat ahead of the front edge of the same polypeptide when

Fig. 1. Results of SDS/polyacrylamide-gel electrophoresis [Laemmli (1970) system] of rat liver microsomal membrane fractions from variously treated rats

The gel was stained with Coomassie Blue. 1 and 10, Control; 2, phenobarbital (two daily doses); 3 and 12, phenobarbital (five daily doses); 4, 2-acetylaminofluorene (6 days); 5, 3-methylcholanthrene (one dose); 6, 3-methylcholanthrene (five daily doses); 7, safrole; 8, isosafrole; 9, polychlorinated biphenyls; 11, pregnenolone-16 α -carbonitrile. The black dots (from top to bottom) on the right hand side of slot 9 indicate the positions of polypeptides I, III, IV, V, VII and VIII respectively; that on the right hand side of slot 8 indicates the position of polypeptide II and that on the right hand side of slot 1 indicates the position of polypeptide VI. The significance of the single white dot in each of slots 3, 6, 7 and 8 is explained in the text. The numbers 45 and 67 in the right hand side of slot 12 indicate the positions

Fig. 2. Results of SDS/polyacrylamide-gel electrophoresis [Laemmli (1970) system] of liver microsomal membrane fractions from rats treated with ethanol and also with various combinations of xenobiotics

The gels were stained with Coomassie Blue. (a) 1, Control (chow diet); 2, control (liquid isocaloric diet); 3, chronic ethanol. The black dot on the left hand side of slot 3 indicates the position of the polypeptide (VI) principally affected by ethanol feeding. (b) 1 and 7, Control; 2, phenobarbital (five daily doses); 3, 3-methylcholanthrene (five daily doses); 4, phenobarbital (three daily doses)+3-methylcholanthrene (three daily doses); 5, polychlorinated biphenyls; 6, 2-acetylaminofluorene (5 days); 8, 2-acetylaminofluorene (5 days) then phenobarbital (three daily doses); 9, 2-acetylaminofluorene (5 days) then 3-methylcholanthrene (three daily doses); 10, 2-acetylaminofluorene (5 days) then phenobarbital (three daily doses)+3-methylcholanthrene (three daily doses); 11, 2-acetylaminofluorene+polychlorinated biphenyls. The black dots (from top to bottom) on the right hand side of slot 9 indicate the positions of polypeptides I-VIII respectively. The arrow indicates the direction of electrophoretic migration; only the areas of the gels between where polypeptides of approx. 43 000 (top) and 75 000 (bottom) mol. wt. migrated are shown, as no changes were detected outside these areas. Results essentially identical with those shown here were obtained from electrophoretic analyses of at least three other specimens (all obtained from individual rats) of each type of sample listed above. The doses of the various agents used are described in the Materials and Methods section.

induced by phenobarbital; this may signify that in the case of induction after administration of pregnenolone-16 α -carbonitrile, polypeptide V was a mixture of at least two components. (6) Polypeptide VI was relatively specifically affected by ethanol treatment. However, when 2-acetylaminofluorene was first given and then followed by phenobarbital+3-methylcholanthrene or polychlorinated biphenyls, examination of the original gels before photography suggested that this polypeptide was also moderately induced by these agents. Interestingly, when phenobarbital+3-methylcholanthrene or polychlorinated biphenyls were given without 2-acetylaminofluorene, they did not appear to exert this effect. Treatments with phenobarbital, polychlorinated biphenyls, safrole, isosafrole and pregnenolone-16 α -carbonitrile all markedly induced polypeptide V and caused it to extend into the areas of the gels occupied by polypeptide VI; this made it difficult on the basis of the present studies to determine whether the latter polypeptide

had been affected by these agents. (7) Polypeptide VII was markedly induced by treatment with 3-methylcholanthrene, polychlorinated biphenyls, safrole and isosafrole. Treatment with benzo[a]pyrene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and β -naphthoflavone (results not shown) also markedly increased the amount of this polypeptide. (8) Polypeptide VIII was markedly induced by the same agents that induced polypeptide VII, with the exception of safrole and isosafrole. With respect to the total number of patterns of induction recognized here, those shown in slots 6 and 7 are considered almost identical, as are those in slots 15 and 16; thus, excluding the two control patterns (slots 1 and 10), some twelve patterns have been identified.

We have attempted, employing the electrophoretic method of Thomas *et al.* (1976), to determine which of the polypeptides affected by the treatments with 3-methylcholanthrene, phenobarbital and 2-acetylaminofluorene used here contained haem. The results

to where standard polypeptides of 45 000 and 67 000 mol. wt. migrated; the arrow and OR on the right hand side of this slot indicate the direction of electrophoretic migration and the origin of the electrophoretogram respectively. Results essentially identical with these shown here were obtained from electrophoretic analyses of at least three other specimens (all obtained from individual rats) of each type of sample listed above. The doses of the various agents used are described in the Materials and Methods section.

Fig. 3. Diagrammatic representation of the patterns of induction of rat liver microsomal membrane polypeptides detected by SDS/polyacrylamide-gel electrophoresis

Each pattern is a composite diagram representing the results from at least four animals. 1, Control (C); 2, phenobarbital (two daily doses) [PB (2)]; 3, phenobarbital (five daily doses) [PB (5)]; 4, 3-methylcholanthrene (one dose) [MC (1)]; 5, 3-methylcholanthrene (five daily doses) [MC (5)]; 6, polychlorinated biphenyls (PCBs); 7, phenobarbital (three daily doses)+3-methylcholanthrene (three daily doses) (PB/MC); 8, safrole (or isosafrole) (SAF); 9, pregnenolone-16 α -carbonitrile (PCN); 10, control for chronic ethanol (liquid isocaloric diet) [C(ET)]; 11, chronic ethanol (21 days) (ET); 12, 2-acetylaminofluorene (AAF); 13, 2-acetylaminofluorene+phenobarbital (three daily doses), (AAF/PB); 14, 2-acetylaminofluorene+3-methylcholanthrene (three daily doses) (AAF/MC); 15, 2-acetylaminofluorene+phenobarbital (three daily doses)+3-methylcholanthrene (three daily doses) (AAF/PB/MC); 16, 2-acetylaminofluorene+polychlorinated biphenyls (AAF/PCBs). The polypeptides are labelled I-VIII on the right and their approx. mol.wts. $\times 10^{-3}$ are indicated on the left. The following functions are assigned to the eight polypeptides shown: I, putative haemopolypeptide; II, minor species of epoxide hydratase (tentative); III, epoxide hydratase; IV, unknown; V, cytochrome P-450; VI, ethanol-inducible polypeptide; VII and VIII, cytochrome P-448-associated. The doses of the various agents are described in the Materials and Methods section.

of a typical experiment of this nature are shown in Fig. 4(a) (haem-staining pattern) and Fig. 4(b) (Coomassie Blue-staining pattern). Two distinct bands (B and C) staining positively for haem are evident in the control specimens. Treatment with 3-methylcholanthrene increased the haem-staining intensity of polypeptide B; interestingly, the polypeptide most markedly increased in amount by treatment with this agent (indicated by the white dot in slot 2 of Fig. 4b) did not stain positively for haem. Administration of phenobarbital principally resulted in a marked broadening of the haem-staining intensity of polypeptide B; examination of several gels suggested that this was in part due to a marked increase in a haemopolypeptide migrating just ahead of polypeptide B, but the resolution achieved and the Coomassie Blue-staining pattern (specifically the polypeptide indicated by the white dot in slot 3 of

Fig. 4b) did not permit a definitive statement to be made about this. The principal effect of treatment with 2-acetylaminofluorene was to markedly increase the staining intensity of haemopolypeptide A, barely detectable in the control or 3-methylcholanthrene-treated fractions; this polypeptide was clearly markedly increased in amount when Coomassie Blue was used as the staining reagent (indicated by the white dot in slot 4 of Fig. 4b). The amount of polypeptide A was also moderately increased after phenobarbital administration, both as evaluated by haem-staining and staining with Coomassie Blue.

After most of the experiments reported here were concluded, Levin *et al.* (1978) reported that treatment of rats with 2-acetylaminofluorene resulted in a marked increase in epoxide hydratase activity; these workers also demonstrated by electrophoresis that administration of this agent resulted in a marked

Fig. 4. Results of SDS/polyacrylamide-gel electrophoresis (system of Thomas *et al.*, 1976) of the haemopolypeptides of liver microsomal membrane fractions from variously treated rats

(a) Gel stained for haemopolypeptides; (b) the same gel stained with Coomassie Blue. 1 and 5, Control; 2 and 6, 3-methylcholanthrene (five daily doses); 3 and 7, phenobarbital (five daily doses); 4 and 8, 2-acetylaminofluorene (6 days). The significance of the polypeptides labelled A, B and C on the left hand side of slot 4 of (a) is explained in the text, as is the significance of the polypeptides indicated by white dots in slots 2, 3 and 4 of (b). Only the area of the gel from the origin (OR) to where a polypeptide of approx. 40000 mol.wt. would migrate is shown. The position to which a polypeptide of approx. 50000 mol.wt. would migrate is indicated on the right hand side of the Figure. The arrow on the right hand side of slot 8 (b) indicates the direction of electrophoretic migration. Results essentially identical with these shown here were obtained from similar electrophoretic analyses of at least three other specimens (all obtained from individual rats) of each type of sample listed above. The doses of the various agents used are described in the Materials and Methods section.

increase in a microsomal membrane polypeptide of approx. 49000 mol.wt. These results suggested that epoxide hydratase might be one of the two polypeptides (cf. Fig. 1, slot 4) found to be markedly increased in amount after treatment with 2-acetylaminofluorene. We thus analysed a sample of purified epoxide hydratase in the systems of both Laemmli (1970) and Thomas *et al.* (1976). The results of an analysis using the former system are shown in Fig. 5. The purified enzyme preparation is seen to contain two polypeptides, each migrating fractionally slower than polypeptides I and III. No other polypeptides were detectable in the enzyme preparation. We reasoned that the slight difference in migration seen between the two polypeptides present in the enzyme preparation and between membrane polypeptides I and III might be due to the presence of the detergent used for purification (Lubrol 12A-9) in the former. Accordingly, it seemed important to perform mixing experiments in which samples of the purified enzyme were added to samples of control and 2-acetylaminofluorene-treated microsomal fractions to test whether the polypeptides in the enzyme preparation co-electrophoresed with certain of the membrane polypeptides.

The results of analysis of such mixtures are shown in slots 3 and 7 (control microsomal fraction plus enzyme) and 5 (2-acetylaminofluorene-treated microsomal fraction plus enzyme) respectively. When compared with the patterns of control (slots 1 and 8) and 2-acetylaminofluorene-treated microsomal fractions (slot 6), each with no enzyme added, the cumulative results of these analyses indicated that the two polypeptides present in the purified epoxide hydratase preparation did co-electrophorese with polypeptides I and III respectively. When analysed in the system of Thomas *et al.* (1976) (results not shown), the enzyme preparation exhibited one principal polypeptide, which corresponded exactly in migration (mol.wt. approx. 49000) to that found to be markedly induced by treatment with 2-acetylaminofluorene (cf. slot 4 of Fig. 4b). A lesser amount of a polypeptide of approx. 100000 mol.wt. was also visible. Neither of

Fig. 5. Results of SDS/polyacrylamide-gel electrophoresis [Laemmli (1970) system] of purified epoxide hydratase and various microsomal membrane fractions

The gel was stained with Coomassie Blue. 1 and 8, Control microsomal fraction; 2 and 4, epoxide hydratase preparation; 3 and 7, control microsomal fraction+epoxide hydratase; 5, 2-acetylaminofluorene-treated microsomal fraction+epoxide hydratase; 6, 2-acetylaminofluorene-treated microsomal fraction. Approx. $0.67 \mu\text{g}$ of the epoxide hydratase preparation was added to slots 2, 3, 4 and 5; approx. $0.94 \mu\text{g}$ of the enzyme preparation was added to slot 7. Approx. $25 \mu\text{g}$ of microsomal protein was added to slots 1, 3, 5, 6, 7 and 8. The direction of migration is indicated by the arrow. Only the area of the gel from the origin to where a polypeptide of approx. mol.wt. 35000 would migrate is shown. The position to which a polypeptide of approx. 50000 mol.wt. would migrate is indicated on the right hand side of the Figure. Polypeptides I and III are indicated by the white dots in slot 6. Results essentially identical with those shown here were obtained in two other electrophoretic analyses of the same samples.

these two polypeptides stained positively for haem, under the conditions tested. It should be noted that the enzyme preparation was purified by using relatively strong detergent treatment (1% Lubrol 12A-9) and both anion- and cation-exchange chromatography (cf. Knowles & Burchell, 1977); it is possible

that haem could become detached from a haemopolypeptide under these conditions.

Discussion

These results illustrate a complexity of responses of rat liver microsomal membrane polypeptides to administration of xenobiotics that appreciably exceeds that previously recognized by SDS/polyacrylamide-gel electrophoresis. The greater complexity stems principally from the demonstration that a group of eight polypeptides of 47000–56000 mol.wt. was involved in the response to the various agents employed. Our previous study (Cameron *et al.*, 1976a) showed that two polypeptides (I and III) were induced by treatment with 2-acetylaminofluorene and that these differed from the principal polypeptide (V) induced by phenobarbital and from the two major polypeptides (VII and VIII) induced by treatment with 3-methylcholanthrene or benzo[a]pyrene. This work delineates three further members of this group: polypeptides II (induced most markedly by longer-term treatment with 3-methylcholanthrene and by administration of polychlorinated biphenyls), IV (which responded to all of the agents employed, except 3-methylcholanthrene) and VI (induced by ethanol). Previous electrophoretic studies (cf. Levin *et al.*, 1978; Schut & Thorgeirsson, 1978) appear to have resolved only four or five polypeptides in the mol.wt. range 47000–56000. An additional feature, demonstrated by using certain combinations of agents, is that all eight polypeptides may be induced simultaneously (cf. Figs. 2b and 3). Although twelve different induction patterns are presented here, it is apparent that if polypeptides I–VIII can be regulated independently, as the present results seem to suggest, a substantially greater number of patterns of induction involving just these components is theoretically possible. It is probable that application of other techniques (e.g. isoelectric focusing; cf. Warner *et al.*, 1978) will permit further resolution of these particular polypeptides. However, the demonstration that at least eight polypeptides are involved provides a minimal baseline for comparative purposes in future studies of the present nature using SDS/polyacrylamide-gel electrophoresis.

An important consideration is the possible functional activities associated with polypeptides I–VIII. In previous studies, electrophoresis of purified cytochrome P-450 and P-448 species from rat liver has demonstrated that they have mol.wts. ranging from 44000 to 56000 (cf. Ryan *et al.*, 1977). The work described here using haem staining suggests that a minimum of three haemopolypeptides (Fig. 4a) is detectable in the area in which polypeptides I–VIII migrate. However, it is apparent that it is not possible to correlate directly the polypeptide patterns obtained with the system of Laemmli (1970) with those ob-

tained with the system of Thomas *et al.* (1976) (cf. Figs. 1 and 4). This is primarily due to the different buffers and SDS concentrations used in the two systems and to omission of mercaptoethanol during sample solubilization in the latter system. Despite these considerations, it appears justifiable (cf. Alvares & Siekevitz, 1973) to state that polypeptide V is the principal phenobarbital-responsive species of cytochrome *P*-450 and that at least one of polypeptides VII and VIII represents cytochrome *P*-448 (see below). Comments on the possible identities of polypeptides I-III are made subsequently. Chronic ethanol feeding has been reported to increase the amounts of two polypeptides of 53400 and 52000 mol.wt. (Ohnishi & Lieber, 1977). Polypeptide VI of approx. 53000 mol.wt. appears to have a special relationship to ethanol (cf. Villeneuve *et al.*, 1976), although our studies to date have not directly demonstrated that it is a haemopolypeptide. We are not able to assign a function to polypeptide IV at the present time.

An important finding to emerge from this work has been that concerning polypeptides VII and VIII and their relationship to agents that induce cytochrome *P*-448. Our studies have shown that both of these polypeptides are induced by administration of 3-methylcholanthrene, benzo[*a*]pyrene, polychlorinated biphenyls, β -naphthoflavone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, all potent inducers of cytochrome *P*-448. Recent work in our laboratories (R. N. Sharma, R. G. Cameron & R. K. Murray, unpublished work) has revealed that three major carcinogenic polycyclic aromatic hydrocarbons, namely 7,12-dimethylbenz[*a*]anthracene, benz[*a*]anthracene and dibenz[*a,h*]anthracene, also induce both polypeptides VII and VIII. Dickens *et al.* (1978) have demonstrated by electrophoresis that administration of isosafrole to rats induces a novel polypeptide of 54000 mol.wt.; other studies indicated that this was probably a haemopolypeptide. Our observations confirm the electrophoretic findings of these workers and also show that safrole induces the same polypeptide (VII). Of particular interest is our finding that polypeptide VII alone is induced by safrole or isosafrole, whereas the inducers of cytochrome *P*-448 increase the amounts of both polypeptides VII and VIII. As isosafrole-induced microsomal fractions exhibit different spectral properties from 3-methylcholanthrene-induced microsomal fractions (Dickens *et al.*, 1978), it appears possible that polypeptide VIII may be the species principally involved in cytochrome *P*-448 activity.

Within several days after exposure to 2-acetylaminofluorene and certain hepatocarcinogenic azo dyes and nitrosamines, striking increases in polypeptides I and III are detectable (Cameron *et al.*, 1976a). Cameron *et al.* (1977) have also reported that both polypeptides I and III persist in increased

amounts in 2-acetylaminofluorene-induced hyperplastic nodules and hepatomas. The results of Levin *et al.* (1978), showing that epoxide hydratase (approx. 49000 mol.wt.) is induced after administration of 2-acetylaminofluorene to rats, have been referred to above. These workers also found that epoxide hydratase was present in increased amounts in the microsomal fractions of 2-acetylaminofluorene-induced hyperplastic nodules and hepatomas and they showed that it corresponded to a pre-neoplastic antigen (cf. Okita & Farber, 1976; Griffin & Kizer, 1978). We have recently confirmed that administration of 2-acetylaminofluorene to rats results in a marked increase in epoxide hydratase activity and that this enzyme is also elevated in both hyperplastic nodules and hepatomas resulting from treatment with this agent (R. G. Cameron & H. L. Gurtoo, unpublished work). In this study we have made a number of observations concerning polypeptides I and III and also their possible relationship to epoxide hydratase. We shall interpret these findings in the light of the following considerations. Our analyses using the Laemmli system have consistently revealed that both polypeptides I and III are induced after administration of 2-acetylaminofluorene (this statement is based on observations made from analyses of individual microsomal fractions from approx. 50 rats treated with this agent in the manner described in the Materials and Methods section). We believe that we detect these two polypeptides [instead of the one reported by Levin *et al.* (1978)] because of the relatively superior power of the electrophoretic system used here; the fact that 10% polyacrylamide separating gels were used in this study, whereas 7% gels were used in the study of Levin *et al.* (1978), may be relevant to this matter. On the other hand, these two polypeptides do not separate in the gel system of Thomas *et al.* (1976), in which they migrate together as the polypeptide zone (staining positively for haem) indicated in slot 4 of Fig. 4(b). The results obtained from analysis of the purified epoxide hydratase (Fig. 5) suggest that at least one of polypeptides I and III corresponds to this enzyme. Thus our results may be interpreted in three ways: (i) 2-acetylaminofluorene (and certain other agents) induces both epoxide hydratase and a novel haemopolypeptide (?mono-oxygenase species); (ii) these agents induce two species or subunits of epoxide hydratase, one of which binds haem; (iii) these agents induce epoxide hydratase and a second polypeptide with a high avidity for haem. We favour the first interpretation and tentatively designate polypeptide I as the putative haemopolypeptide [because detachment of haem in the Laemmli (1970) system would lower its molecular weight], with polypeptide III representing the principal species of epoxide hydratase from control rat liver. In this connection, Malejka-Giganti & McIver (1978) have confirmed our original claim (Cameron *et al.*, 1976a)

that 2-acetylaminofluorene induces a novel haemopolypeptide; however, neither their work nor the present has provided experimental evidence that would eliminate the two other possibilities described here. With respect to the first possibility, if one of the two polypeptides in the epoxide hydratase preparation is indeed the apopolypeptide moiety of the putative haemopolypeptide, it is puzzling that this polypeptide should co-purify with epoxide hydratase. However, the observation that a higher-molecular-weight component, possibly a dimer of the two polypeptides, was noted in the system of Thomas *et al.* (1976) could be interpreted to indicate that the two polypeptides may interact quite strongly under mild dissociating conditions. Unequivocal distinction between the three possibilities listed above may have important implications for the understanding of the metabolism of certain hepatocarcinogens, because if the first possibility is correct, the novel haemopolypeptide species could play a role in the conversion of such compounds to active and/or inactive metabolites. As discussed by Levin *et al.* (1978), it is intriguing that no role for epoxide hydratase in the metabolism of 2-acetylaminofluorene has yet been shown; however, the participation of mono-oxygenase species in its metabolism appears to be substantiated (Miller & Miller, 1974). In addition, resolution of the exact nature of these polypeptides has implications for knowledge of the number of species (or subunits) of epoxide hydratase present in rat liver, for understanding the nature of pre-neoplastic antigens and also possibly for the elucidation of the mechanism of resistance to the cytotoxic effects of hepatocarcinogens exhibited by certain pre-neoplastic cells (Cameron *et al.*, 1976b).

It is also pertinent to comment that certain other agents used here caused a moderate induction of polypeptides I and III. Bresnick *et al.* (1977) have shown that both phenobarbital and 3-methylcholanthrene treatment of rats (in protocols comparable with these used here) induce this enzyme. These workers demonstrated that the effect of 3-methylcholanthrene was less marked than that of phenobarbital and that the enzyme induced by this agent exhibited a more restricted specificity for oxides. Fig. 3 demonstrates that longer-term administration of phenobarbital increased the amount of polypeptide III quite markedly. In contrast, longer-term administration of 3-methylcholanthrene resulted in a notable increase of polypeptide II. We thus suggest that polypeptide II may represent another species of epoxide hydratase. Of additional interest is that administration of phenobarbital and of polychlorinated biphenyls also increased the amount of this polypeptide, in addition to their effects on polypeptide III. Both of these latter agents also resulted in moderate increases of the putative haemopolypeptide (I). Regardless of the precise catalytic functions of polypeptides I-III, the

abilities of the above agents to induce one or more of these polypeptides could affect the metabolic capacities of microsomal fractions of animals treated with these particular xenobiotics. This may be of importance for understanding the results of experiments in which phenobarbital or 3-methylcholanthrene are used to modulate the hepatocarcinogenicity of various compounds (cf. Wattenberg, 1978) and also for helping to account for the mutagen-forming capacities of microsomal fractions used in the Ames assay (McCann *et al.*, 1975).

Since this paper was initially submitted, we have analysed six more individual preparations of epoxide hydratase. All of them contained both of the polypeptides described above. However, certain of the preparations stained positively for haem. It thus appears possible to purify both epoxide hydratase and the haem-staining polypeptide.

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