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The detection of enzyme induction by rat liver microsomes prepared by isoelectric precipitation

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A comparison was made of various indices of the hepatic drug-metabolizing apparatus associated with the postmitochondrial supernatant, microsomes harvested by differential centrifugation, and microsomes harvested by isoelectric precipitation from control, phenobarbitone-pretreated and 3-methylcholanthrene-pretreated rats. The metabolic capabilities and distinctions between control and induced rats for each of the three liver preparations compared favourably as determined by the concentration of cytochrome b_5 and P-450 and by the activity of NADPH-cytochrome c reductase, 4-dimethylaminoantipyrine *N*-demethylase and aryl hydrocarbon (benzo[a]pyrene) hydroxylase. The results suggest that the relatively simple isoelectric precipitation technique is a useful alternative to the conventional method of differential centrifugation for the preparation of hepatic microsomes.

Hepatic microsomes, which form by fragmentation and vesiculation of the endoplasmic reticulum during homogenization of the liver, contain a highly complex mixture of enzyme activities. This mixture includes the microsomal monooxygenases which are involved in the oxidation of both endogenous and exogenous chemical substrates to give more hydrophilic metabolic products. Mechanistic studies have confirmed or have implicated the formation of metabolically active arene oxide intermediates in this process and these reactive species are associated with cellular toxicosis (Parke 1972; Gillette 1975; Daly et al 1972; Jerina & Daly 1974). The investigation of the microsomal-mediated metabolism of chemical substrates is routinely carried out by incubating the chemical and the required cofactors with either the hepatic postmitochondrial supernatant (PMS) fraction or with a microsomal fraction obtained by further differential centrifugation (MDC) of the crude postmitochondrial supernatant.

Microsomes have long been known to aggregate under acidic conditions (Claude 1946a, b) and based on this fact these vesicles can be prepared by a simple and rapid isoelectric precipitation technique which obviates the need for a high speed centrifugation step. There is, however, a paucity of information on the properties of microsomes prepared by isoelectric precipitation (MIP). There are a few reports demonstrating the similarity between the basal enzyme activities in microsomes harvested by differential centrifugation and isoelectric precipitation (Karler & Turkanis 1968; Mitchard 1970; Fry & Bridges 1974) but, to the best of our knowledge, no reports pertaining to the induced enzyme activities in these microsomal preparations.

This paper describes a comparison of certain criteria related to the drug-metabolizing apparatus associated with the hepatic postmitochondrial supernatant, microsomes harvested by isoelectric precipitation and microsomes harvested by differential centrifugation. These criteria include the concentration of protein, cytochrome b_5, cytochrome P-450 and the activity of NADPH-cytochrome c reductase, 4-dimethylaminoantipyrine *N*-demethylase and benzo[a]pyrene hydroxylase.

These criteria were employed to test the ability of the PMS, MIP and MDC to discern between control animals and animals pretreated with phenobarbitone (PB) and 3-methylcholanthrene (3-MC). It has been shown that certain in vitro conditions (such as the concentration of microsomal protein) can influence the detection of microsomal enzyme induction (Fouts 1970). It was decided, therefore, to examine whether the three liver preparations, in which the various components of the drug-metabolizing apparatus are purified to different extents, were equally sensitive to induction of cytochrome P-450 monooxygenases (by PB) and of cytochrome P-448 (P_1-450) mono-oxygenases (by 3-MC).

MATERIALS AND METHODS

Materials Benzo[a]pyrene, 3-methylcholanthrene, cytochrome c (horse heart, type III), $NADP^+$ (monosodium salt), α-D-glucose-6-phosphate and α-D-glucose-6-phosphate dehydrogenase (Baker's

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yeast) were supplied by Sigma Chemical Company; 4-dimethylaminoantipyrine by Aldrich; SKF 525A by Smith Kline and French, and sodium phenobarbitone by the Ontario Veterinary College, Guelph. [^{3}H]benzo[a]pyrene was prepared by palladium-catalysed tritium exchange in trifluoroacetic acid (New England Nuclear) and purified by thin layer chromatography (t.l.c.).

Methods. Animal treatment. Four groups of nine male Wistar rats (60 days old) had free access to Purina Rat Chow and water. After three days of acclimatization to a 12 h diurnal light regimen, each group was injected intraperitoneally on two successive days at 9 a.m. (i.e. the beginning of the light period) with one of the following: sodium phenobarbitone (300 μmol [76 mg] kg^{-1}) dissolved in isotonic saline; 3-methylcholanthrene (100 μmol [27 mg] kg^{-1}) dissolved in corn oil; or one of the two vehicles (0·9% NaCl or corn oil) alone. Following the second administration all animals were fasted for 24 h to lower liver glycogen levels before being killed by cervical dislocation.

Isolation of microsomes. Livers were removed and immediately transferred to ice-cold Kreb-Ringer solution. Samples (5 g) from three livers were combined. This gave twelve pooled livers which were homogenized in sucrose-EDTA (0·25M–0·1mM) and centrifuged at 10 000 *g* for 20 min at 4 °C. The pellet was resuspended and re-centrifuged before discarding it. The first and second postmitochondrial supernatants were combined, adjusted to 60 ml (25% suspension) and divided into three 20 ml aliquots. One aliquot was stored at 4 °C and represented the postmitochondrial supernatant (PMS). Another aliquot was centrifuged at 100 000 *g* for 90 min at 4 °C on a sucrose-EDTA cushion (5 ml, 1·6M-0·1mM). The microsomal band was resuspended in 20 ml sucrose-EDTA solution and represented microsomes prepared by differential centrifugation (MDC). The remaining aliquot was acidified to pH 5·4 with several drops of acetate buffer (0·2M, pH 4·0) and centrifuged at 10 000 *g* for 10 min at 4 °C. The pellet was washed once in sucrose (0·25M)-EDTA (0·1 mM): glycerol (4 : 1, v/v) and re-centrifuged. The microsomal pellet was resuspended in 20 ml sucrose-EDTA solution and represented microsomes prepared by isoelectric precipitation (MIP).

Assay procedures. Protein (Lowry et al 1951), cytochrome b_5 (Raw & Mahler 1959) and cytochrome P-450 content (Omura & Sato 1964a, b) and NADPH-cytochrome c reductase activity (Williams & Kamin 1962) were measured as described except the millimolar extinction coefficient of cytochrome b_5 was taken to be 185 cm^{-1} mM^{-1} (Omura & Sato 1964a).

The activity of benzo[a]pyrene hydroxylase was assayed essentially as described (DePierre et al 1975; Nesnow et al 1977) with the following modifications. An NADPH-regenerating system (0·5mM $NADP^+$, 4·5 mM α-D-glucose-6-phosphate and, except for PMS, 0·33 U ml^{-1} of glucose 6-phosphate dehydrogenase) was employed; for which reason the assays were pre-incubated for 15 min and begun by the addition of substrate at 15 s intervals. By monitoring the increase in absorbance at 340 nm using difference spectroscopy, the PMS, to which no glucose-6-phosphate dehydrogenase was added, was shown to contain sufficient endogenous glucose-6-phosphate dehydrogenase activity to generate an adequate supply of NADPH. The benzo[a]pyrene hydroxylase assay was performed in Tris-HCl buffer (17mM, pH 7·5). The incubation time was extended from 15 to 30 min and for this reason the concentration of benzo[a]pyrene was raised from 60 to 200 μM.

The rate of oxidative *N*-demethylation of 4-dimethylaminoantipyrine (DMAP) was measured by quantifying the production of formaldehyde. Unless otherwise indicated the 3 ml incubation mixtures contained PMS, MIP or MDC protein, DMAP (20 mM), Tris HCl buffer (50 mM, pH 8·0), $MgCl_2$ (4·2 mM), semicarbazide HCl (5·0 mM), α-D-glucose-6-phosphate (6·7 mM), $NADP^+$ (0·5 mM) and, except for PMS, 0·33 U ml^{-1} glucose-6-phosphate dehydrogenase. Standards contained a known amount of formaldehyde in place of the substrate and zero-time incubations served as blanks.

The assays were pre-incubated by shaking in a water bath at 37 °C for 15 min. The reactions were started by the addition of substrate at 15 s intervals and stopped after 10 min by the addition of equal volumes (0·5 ml) of 25% $ZnSO_4.7H_2O$ and saturated $Ba(OH)_2$. After centrifugation, the formaldehyde semicarbazone present in 2 ml of the clear supernatant was developed in double strength Nash reagent (Nash 1953) at 45 °C and the absorbance read at 412 nm.

In a series of separate experiments to examine the design of the *N*-demethylase assay, a K_m value for control ($7·2 \times 10^{-4}$ M) and optimum conditions (pH 8·0, 10 min incubation period, 20 mM substrate concentration with less than 3 mg protein ml^{-1}) were observed which closely resemble those described (Dewaide & Henderson 1968). Other characteristics of oxidative *N*-demethylation, such as non-linear reciprocal kinetic plots (Pederson & Aust 1970; Poland & Nebert 1973) and the preferential inhibi-

tion by SKF (10^{-6} to 10^{-4} M) of the *N*-demethylase activity in microsomes from phenobarbitone-pretreated rats over the activity in microsomes from 3-methylcholanthrene rats (Pederson & Aust 1970; Sladek & Mannering 1966) were also observed (unpublished results).

In all assays protein concentrations of less than 2 mg ml^{-1} (2 mg ml^{-1} for PMS, 1 mg ml^{-1} for MIP and MDC) were used to maximize the difference between control and induced activities (Fouts 1970).

RESULTS

The results obtained for control (corn oil-pretreated), phenobarbitone-pretreated and 3-methylcholanthrene-pretreated rats are summarized in Tables 1, 2 and 3 respectively. To avoid repetition the saline control data, being identical to the corn oil control data, has been omitted.

Table 1. A comparison of certain drug-metabolizing parameters of the PMS, MIP and MDC from control rats. Values are mean (with s.d.)

Postmitochondrial supernatant (PMS)	Microsomes from isoelectric precipitation (MIP)	Microsomes from differential centrifugation (MDC)
Protein (mg g^{-1} liver)		
73·4 (3·4)	36·2 (1·0)	25·4 (3·7)
Cytochrome b_5 (nmol g^{-1} liver)		
4·97 (0·12)	4·83 (0·21)	4·80 (0·20)
Cytochrome P-450 (nmol g^{-1} liver)		
14·3 (2·5)	12·3 (1·5)	11·3 (0·6)
NADPH-Cytochrome c reductase (μmole Cyt. c reduced g^{-1} liver h^{-1})		
172 (12)	189 (4)	153 (5)
4-Dimethylaminoantipyrine *N*-demethylase (μmol HC-O g^{-1} liver h^{-1})		
14·0 (0·4)	16·9 (0·4)	11·2 (0·6)
Benzo[a]pyrene hydroxylase (nmol B[a]P hydroxylated g^{-1} liver min^{-1})		
6·30 (1·81)	6·58 (0·36)	6·98 (1·96)

From Table 1 it is evident that the MDC, which contains the least amount of protein, is the most purified and, hence, least complexed of the three liver preparations. The MIP is associated with between 40 to 45% more protein than the MDC; a value which coincides with that previously reported (Fry & Bridges 1974).

To illustrate the fact that protein content is inversely proportional to specific activity, the results are related to the cytochrome content and enzyme activity present in a gram of liver rather than in a mg of protein. Expressed in this way, the results strongly suggest that the absolute cytochrome b_5 and cytochrome P-450 content and the absolute activity of NADPH-cytochrome c reductase, DMAP *N*-demethylase and benzo[a]pyrene hydroxylase are equivalent in all three liver preparations.

Pretreatment with phenobarbitone increased the concentration of cytochrome b_5 and cytochrome P-450 and stimulated the activity of NADPH-cytochrome c reductase and DMAP *N*-demethylase. In contrast to previous reports, phenobarbitone was found to have no effect on benzo[a]pyrene hydroxylase activity (Alvares et al 1968).

Table 2. A comparison of certain drug-metabolizing parameters of the PMS, MIP and MDC from phenobarbitone-pretreated rats. Values are mean (with s.d.) Numbers in [ ] represent increases of test/control.

Postmitochondrial supernatant (PMS)	Microsomes from isoelectric precipitation (MIP)	Microsomes from differential centrifugation (MDC)
Protein (mg g^{-1} liver)		
91·5 (10·3) [1·2]	34·0 (2·1)	28·2 (3·7)
Cytochrome b_5 (nmol g^{-1} liver)		
8·33 (1·35) [1·7]	6·47 (0·80) [1·3]	7·57 (0·51) [1·6]
Cytochrome P-450 (nmol g^{-1} liver)		
30·0 (1·0) [2·1]	24·3 (2·5) [2·0]	27·6 (1·2) [2·2]
NADPH-Cytochrome c reductase (μmol Cyt. c reduced g^{-1} liver h^{-1})		
460 (28) [2·7]	439 (37) [2·3]	360 (8) [2·4]
4-Dimethylaminoantipyrine *N*-demethylase (μmol HCHO g^{-1} liver h^{-1})		
28·5 (0·9) [2·1]	26·3 (2·6) [1·6]	24·5 (1·0) [2·2]
Benzo[a]pyrene hydroxylase (nmole B[a]P hydroxylated g^{-1} liver min^{-1})		
6·20 (2·11)	6·82 (0·66)	7·08 (1·19)

Pretreatment with 3-methylcholanthrene increased the concentration of cytochrome b_5 and cytochrome P-450 (the latter being associated with an inconsistent but characteristic 2 nm shift in the CO-binding spectrum [Alvares et al 1967]) and stimulated the activities of NADPH-cytochrome c reductase and DMAP *N*-demethylase; although the increases were less than those observed following phenobarbitone pretreatment. Unlike phenobarbitone, pretreatment with 3-methylcholanthrene stimulated benzo[a]-pyrene hydroxylase activity by 5–7 fold.

Table 3. A comparison of certain drug-metabolizing parameters of the PMS, MIP and MDC from 3-methylcholanthrene-pretreated rats. Values are mean (with s.d.) Numbers in [ ] represent increases of test/control.

Postmitochondrial supernatant (PMS)	Microsomes from isoelectric precipitation (MIP)	Microsomes from differential centrifugation (MDC)
Protein (mg g^{-1} liver)		
81·0 (10·8) [1·1]	35·1 (1·5)	25·9 (2·8)
Cytochrome b_5 (nmol g^{-1} liver)		
6·86 (0·45) [1·4]	6·30 (0·96) [1·3]	6·57 (0·58) [1·4]
Cytochrome P-450 (nmol g^{-1} liver)		
22·3 (0·6) [1·6]	17·3 (0·6) [1·4]	18·6 (1·5) [1·7]
NADPH-Cytochrome c reductase (μmol Cyt. c reduced g^{-1} liver h^{-1})		
303 (17) [1·8]	489 (44) [2·6]	357 (14) [2·3]
4-Dimethylaminoantipyrine-*N*-demethylase (μmol HCHO g^{-1} liver h^{-1})		
16·8 (0·3) [1·2]	19·6 (1·2) [1·2]	13·8 (1·5) [1·2]
Benzo[a]pyrene hydroxylase (nmol B[a]P hydroxylated g^{-1} liver min^{-1})		
42·6 (4·2) [6·8]	40·8 (1·4) [6·2]	40·0 (3·0) [5·7]

The results indicate no major differences between the ability of the three liver preparations to detect increases in microsomal drug-metabolizing activity following induction by phenobarbitone and 3-methylcholanthrene.

DISCUSSION

The in vitro metabolism of xenobiotics commonly employs the hepatic postmitochondrial supernatant or hepatic microsomes harvested by differential centrifugation. This paper demonstrates that the absolute drug-metabolizing capability of hepatic microsomes harvested by isoelectric precipitation is commensurate with both of these conventional liver preparations.

In addition to confirming the similarity between PMS, MIP and MDC, this paper describes, we believe for the first time, the equal ability of these three liver preparations to monitor the effects of pretreatment with the cytochrome P-450 inducer, phenobarbitone, and the cytochrome P-448 inducer, 3-methylcholanthrene.

Whilst the in vitro detection of microsomal induction is reported to be partly dependent on the concentration of microsomal protein (Fouts 1970), the results obtained demonstrate that the additional protein associated with the microsomes prepared by isoelectric precipitation fails to adversely influence the detection of induction with the MIP preparation.

It is concluded that the preparation of microsomes by isoelectric precipitation affords a liver fraction which in its absolute drug-metabolizing capabilities and in its ability to discern between control rats and rats pretreated with phenobarbitone and 3-methylcholanthrene is commensurate with microsomes prepared by differential centrifugation. Thus, isoelectric precipitation, being simple, quick and obviating the need for high-spin centrifugation, is an attractive method of harvesting liver microsomes and is particularly well suited to the preparation of microsomes from large numbers of experimental animals, e.g. in determining structural-activity relationships or dose-response curves for microsomal enzyme inducers. There are occasions, however, when the higher specific activity of microsomes prepared by differential centrifugation may confer certain advantages e.g. in such studies as the in vitro formation of covalent substrate-macromolecular adducts.

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