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THE ASSAY OF MICROSOMAL EPOXIDE HYDROLASE IN NORMAL AND PATHOLOGICAL HUMAN LIVER

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Abstract—Samples of normal and pathological human liver were assayed for microsomal epoxide hydrolase activity using the chlorinated epoxide HEOM§ as substrate. The enzyme activity in liver samples from normal patients was 72.3 ± 9.8 nmoles mg protein^{-1}·min^{-1}, which is comparable to the highest values recorded in mammals, and much higher than those found in rats and mice. A microassay procedure was developed to enable the estimation of activity in needle biopsy samples (5–20 mg), obtained from patients with normal and diseased livers. Three epileptic patients who had received regular doses of phenobarbitone and/or phenytoin showed activities significantly higher than those found in all other samples ($P < 0.01$). These appeared to be examples of enzyme induction. Assays were also performed on samples from six patients with alcohol-related liver disease and one patient with hepatocellular carcinoma. The role of epoxide hydrolase in the metabolism of carcinogenic epoxides is discussed in relation to these findings.

Hepatic microsomal epoxide hydrolase can hydrate a wide variety of exogenous and endogenous epoxides including steroid oxides, insect juvenile hormone analogues, and certain chlorinated insecticides [1–3]. Interest in this enzyme has grown with the discovery that it can hydrate the active metabolites of such carcinogens as benzo[*a*]pyrene [2] and aflatoxin B_1 [4]. Because the normal substrate(s) of this enzyme are unknown, there has been uncertainty about the most appropriate substrates to employ in assay procedures. Radiometric assays have been developed employing as substrates styrene oxide [5], benzo[*a*]pyrene-4,5-oxide [6], octene oxide [7] and 16α, 17α-epoxy-1,3,5(10)-estratren-3-ol, 'estroxide' [8]. A sensitive and specific gas chromatographic assay has been developed which uses as substrate the chlorinated epoxide HEOM, and depends upon electron-capture detection [9]. As normally employed 1 g or more of liver is required for this assay.

The aim of the present investigation was to establish the levels of epoxide hydrolase in normal human liver, and then to make comparisons with the levels in abnormal livers. Since most of the samples were obtained by needle biopsy, and were only 5–20 mg in weight, it was necessary to develop a microassay method. The method chosen was the HEOM assay, on account of its sensitivity and specificity.

MATERIALS AND METHODS

Liver samples. Samples of human liver were obtained from patients undergoing laparoscopy or abdominal surgery under general anaesthesia at Charing Cross Hospital. There were 4 males and 3 females all Caucasian (aged 29–67) with normal livers, 6 alcoholics, 3 female to male trans-sexuals taking methyl testosterone, 3 epileptics on anticonvulsant drugs and single patients with fatty liver of obesity and carcinoma of the bile duct with obstructive jaundice. In the case of one patient with hepatocellular carcinoma, samples were removed within one hour following death.

Liver tissue was obtained either by surgical wedge biopsy or by needle biopsy. The fresh samples were placed in small vials containing 1.15% KCl solution and held at 0–4°. They were transported to Reading for assay, which commenced within 30 hr of sampling. Some samples were stored under various conditions to investigate loss of enzyme activity and for these the first microsomal preparation was commenced in all cases 1.5–2.5 hr after they had been taken. Details of drug therapy and preoperative medication were obtained from hospital records.

Livers were taken from two strains of laboratory mouse—CFLP (Anglia Laboratory Animals, Alconbury, U.K.) and LACG (MRC Laboratory Animals Centre, Carshalton, U.K.).

Wedge samples of human liver (0.5–1 g) and samples of mouse liver were assayed as previously described [9]. Micro samples (5–20 mg) were obtained either by needle biopsy or by removing needle-sized pieces from liver wedges (human) or whole livers (mice). The procedure for assaying them was as follows.

Micro assay procedures. Unlabelled and ^{14}C-labelled HEOM and unlabelled HEOM diol were prepared as described elsewhere [10]. Purity was checked by gas chromatography and radio counting. Unlabelled HEOM was > 99% pure, and the radiochemical purity of [^{14}C]HEOM was > 99%. Solvents and reagents were of ANALAR grade or equivalent unless otherwise stated. Hexane and acetone were

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§ HEOM: 1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methonaphthalene.

redistilled, and examined by gas chromatography before use. Trimethylchlorosilane and hexamethyldisilazane were supplied by Koch-Light Laboratories (Colnbrook, U.K.). Pyridine (spectroscopic grade) was stored over calcium hydride. The silylating mixture was prepared by mixing trimethylchlorosilane (0.5 ml) and hexamethyldisilazane (1 ml) with 5 ml pyridine in a dry test tube.

Liver samples (5–20 mg) were homogenised by hand in ice-cold 1.15% KCl solution using a Jencons all-glass homogeniser to give a final volume of 5 ml. This homogenate was centrifuged for 30 min at 11,000 *g*. The 11,000 *g* supernatant was then spun for 1 hr at 105,000 *g*. The microsomal precipitate so formed was resuspended in 1.15% KCl with the aid of a sonicator before spinning again at 105,000 *g* for 30 min to yield washed microsomes. Using a sonicator, these were then resuspended in reaction medium to give a final volume of 2 ml. An MSE 800 watt sonicator with probe was used, and each sample was treated 4–6 times for periods of 5 sec duration, interspersed with breaks of 15–30 sec to ensure that the preparation did not rise above 4° in temperature. The reaction medium was a phosphate buffer solution, pH 7.4, which gave a final ionic strength of 0.1 M after combination with microsomal suspension.

The incubation procedure was based on the method described by Craven *et al.* [9] for the assay of HEOM hydrolase on the macro scale. Reaction medium (0.5 ml) and microsomal suspension (0.5 ml out of 2 ml) were added to a 10 ml glass-stoppered tube, and this was pre-incubated on a water bath at 37° for 90 sec before adding 10 μl of 2 mg ml^{-1} HEOM in ethanol [= 20 μg HEOM]. After incubation for various periods the reaction was stopped by extracting with 3 ml of diethyl ether. (The assay period routinely used was 30 min.) Two further ether extractions were performed, and the extracts were combined and made up to 10 ml before drying over anhydrous sodium sulphate. Aliquots of ether extract were evaporated to dryness before adding silylating reagent to the residue to convert the *trans* diol to its trimethylsilyl (TMS) derivative. The TMS-diol was analysed in a gas chromatograph fitted with an all-glass SE52 column and an electron-capture detector. The quantity of diol was established by comparing the area of peaks in test extracts with those of TMS-diol standards. The protein content of the microsomal suspension was determined by the method of Lowry *et al.* [11] and specific activities were expressed with respect to microsomal protein. Radiocounting showed that all measurable ^{14}C was removed in the ether extracts with no significant retention in the aqueous phase.

Table 1. Macro and micro assay of epoxide hydrolase activity in human liver—'normal' samples

Sample	Assay procedure	Microsomal protein yield (± S.E.M.)	Epoxide hydrolase activity (nmoles diol mg protein^{-1} min^{-1}) Arithmetic mean (± S.E.M.)	Geometric mean (confidence limits $P < 0.01$)
Test for reproducibility				
Eight needle samples from one liver	Micro	3.18(± 0.23)	42.6(± 1.2)	41.5(36.0–47.7)
Variability of enzyme activities between individuals				
Seven individual liver samples	Macro	5.80(± 0.40)	72.3(± 9.8)	67.6(29.5–155)
assayed by two procedures	Micro	2.86(± 0.30)	31.3(± 5.1)	28.2(9.8–81.4)

All individuals were Caucasian, 4 males, 3 females, 29–67 years of age.
There was no known history of drug intake in any individual.
The micro assays were on average 43.5 ± 4% of the micro assay activities, the range being 33–56%.

RESULTS AND DISCUSSION

Preliminary studies with mouse and human liver microsomes prepared from micro samples of tissue (5–20 mg) confirmed that HEOM hydrolase activity was linear with respect to both protein concentration and time under the previously established assay conditions [9, 10]. Thus 20 μg of HEOM added to < 50 μg of microsomal protein gave a constant rate of diol formation up to 30 min, and this specific

activity was not changed by altering the protein or the substrate concentrations.

Wedge samples of human liver were assayed in two different ways: (1) by the usual macro method [9] and (2) by the micro method, employing 5–20 mg needle samples taken randomly from the wedge. The micro method consistently gave lower values than the macro method for both protein yield and epoxide hydrolase activity. The reason for this is not clear. The difference was not affected by diluting the macro homogenates to the same concentration as the micro ones prior to centrifugation or by resuspending microsomes from needles samples using a glass pestle instead of a sonicator. The procedure for initial homogenisation did not appear to be critical since similar activities were found for duplicate subsamples (0.3–0.5 g) from the same liver which were homogenised with different instruments (Jencons all-glass homogeniser or MSE metal blade homogeniser). The low protein yield may be a consequence of losses incurred during the homogenisation of these very small samples. Notwithstanding this problem, the results obtained by the micro assay are very consistent. When eight subsamples were removed independently from one liver wedge, the standard error for activity was only ± 2.8% (Table 1). The micro method was therefore considered suitable for a comparative study of epoxide hydrolase activity.

A problem encountered with biopsy samples was the risk of loss of enzyme activity during storage. To study this, micro samples were removed from fresh wedges of liver, and stored in small tubes in the presence of 1.15% KCl solution at 4° or −20°. At 4° there was a slow but steady decline in activity, 85%, 79% and 69% of the original activity determined in the freshly sampled liver remaining after 24 hr, 48 hr and 96 hr storage respectively. At −20° the activity was only 61% of the original at 24 hr, but was unchanged after that. It was decided to store samples at 4° in 1.15% KCl solution immediately after taking them, and to assay them as soon as possible. In the subsequent work no samples were stored for longer than 30 hr, and small corrections for loss of activity were made for samples stored for longer than 10 hr, based on the storage data given above.

The seven samples regarded as normal came from patients with normal liver function. No patient had a history of drug therapy that might be expected to affect liver enzymes. The activities in these samples fell within a fairly narrow range (no more than a 3.5-fold difference between the highest and lowest activity by either assay procedure), and gave no evidence of a large difference in epoxide hydrolase activity between sexes or between age groups (Tables 1 and 2). The HEOM-epoxide hydrolase activity found in normal human liver by the macro assay procedure was 72.3 ± 9.8 nmoles mg^{-1} min^{-1}, which is higher than that in most other mammals [3, 10]. The pig and the rabbit gave values of similar magnitude to those of man, but activities were much lower in rats and mice (3–4.6 and 0.5–4 nmoles mg^{-1} min^{-1}). On the other hand rats, mice and men show similar epoxide hydrolase activities when sty-

Table 2. Epoxide hydrolase activities in normal and abnormal samples of human liver*

Liver samples from normal individuals and individuals suffering from alcoholic liver disease

Sample	No. of individuals	Microsomal protein (mg g $liver^{-1}$)(± S.E.M.)	Epoxide hydrolase activity (nmoles mg $protein^{-1}$ min^{-1}) Arithmetic mean	Geometric mean
Normal	7	2.86(± 0.30)	31.3(± 5.1)	28.2(9.8–81.4)†
Alcoholic	6	1.03(± 0.36)	19.8(± 5.0)	19.3(9.7–38.1)†

'Abnormal' liver—individual samples

Sex/age	Description of condition or treatment	Microsomal protein (mg g $liver^{-1}$)	Epoxide hydrolase activity (nmoles mg $protein^{-1}$ min^{-1})
Female/28	Androgens	1.95	3.8
Female/26	Androgens	0.34	36.9
Female/26	Androgens	1.30	16.2
Female/28	Epileptic—receiving regular high dose of phenobarbitone	2.30	78 Macro assay 179
Female/34	Epileptic—receiving regular high dose of phenobarbitone	1.90	186
Female/39	Epileptic—receiving high dose of phenytoin	1.20	138
Female/64	Obesity and fatty liver	0.84	9.3
Male/42	Carcinoma of bile duct	0.50	31 Macro assay 77
Male/53	Post-mortem sample of liver from patient with hepatocellular carcinoma		
	Tumour tissue	1.45	3.2
	Adjacent liver	1.50	9.7

* Determinations by the micro assay method.

† Confidence limits, P = 0.01. The difference between normal and abnormal livers was not significant ($P < 0.1$), using log transformed data.

rene oxide is used as substrate, all values falling within the range of 4–12 nmoles mg^{-1} min^{-1} [2, 3, 12, 13].

With reference to activities determined by the micro assay procedure, these were between 2.6 and 6 times higher in the case of three epileptics who had regularly taken high doses of phenobarbitone and phenytoin than in 'normal' patients (Table 2) and there was no overlap between the two groups. Since the enzyme activities of a population usually show a normal distribution on a log scale, the data were converted to $\log_{10}$ for the purposes of statistical analysis. An analysis of variance was performed upon the logged data, and this showed that the activities of the 'epileptics' were significantly greater than the activities of the 'normal' and 'alcoholic' groups ($P < 0.01$). This suggests that epoxide hydrolase has been induced by regular high doses of phenobarbitone and/or phenytoin. When rats were dosed with phenobarbitone, a 2.5-fold increase in the activity of HEOM-hydrolase was found in liver microsomes, which is of similar magnitude to the apparent levels of induction in these patients [14].

Patients suffering from alcohol-related liver disease showed lower activities than were found in normal livers, but the difference was not statistically significant when activities were expressed in terms of microsomal protein and analysis of variance was conducted, as before, on logged data ($P < 0.1$). It should be noted that this group had lower microsomal protein yields than either the normal group or the epileptics. A likely reason for these relatively low microsomal yields is the diluting effect of either connective tissue and/or fat in these samples. (Neither of these materials should contribute significantly to microsomal protein.) Whatever the reason, the difference between alcoholics and 'normal' samples is substantially increased if activity is expressed in terms of g liver rather than mg microsomal protein and is statistically significant ($P < 0.01$).

In the case of female to male trans-sexuals, two samples fell within the normal range, whereas one had lower activity than this. It is not clear from these figures what were the effects, if any, of the steroid therapy. In theory the steroids could act as inducers of the enzyme, or, if they were carried through to the microsomal preparation, they might act as inhibitors.

The samples from the patient with hepatocellular carcinoma were removed within one hour of death. Hepatic microsomal epoxide hydrolase was found to be fairly stable for 4 hr following death in humans [15] so results from these assays should be comparable to other data. The activity in liver tissue adjacent to the tumour was lower than that found in normal patients, but was 3.1-fold greater than that in the tumour tissue itself. A very similar difference in activity between normal and tumour tissue in humans has been reported by Oesch *et al.* [16] in two cases of bronchiogenic carcinoma (2.9-fold and 3.1-fold greater activity in adjacent tissue). Both observations contrast with the findings of Levin *et al.* [17] who treated rats with 2 acetylaminofluorene. Here the microsomes from hyperplastic nodules or hepatomas were 5–7 times higher in activity than were those from control liver. A precarcinogenic antigen was found to have epoxide hydrolase activity. The question of levels of epoxide hydrolase activity in tumour vs normal tissue awaits clarification.

Many carcinogens including polycyclic aromatic hydrocarbons, aflatoxin B_1, and probably certain chlorinated ethylene derivatives, are converted by microsomal monooxygenase attack into epoxides which can bind to cellular nucleophiles [2, 18]. Thus the metabolism of such epoxides by epoxide hydrolase may have a protective function, and the risk of cellular binding may depend upon the balance between this enzyme and monooxygenase. This balance can be changed by induction, and it would therefore be interesting to know what levels of monooxygenase activity exist in epileptic patients who have relatively high epoxide hydrolase activity.

The hepatic microsomal epoxide hydrolase of humans differs in character from that of rats and mice. This contrast raises serious doubts about the suitability of mice and rats as models for man when testing the carcinogenicity of epoxides, or of compounds which are metabolised to epoxides.

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