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THE APPARENT UBIQUITY OF EPOXIDE HYDRATASE IN RAT ORGANS

FRANZ OESCH*, HANSRUEDI GLATT and HANSUELI SCHMASSMANN†
Section on Biochemical Pharmacology, Institute of Pharmacology, University, Obere Zahlbacher Strasse 67, D-6500 Mainz, West Germany

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Abstract – Using the recently developed sensitive assay with [^{3}H]benzo[a]pyrene 4,5-oxide as substrate, epoxide hydratase was shown to be present in 26 rat (Sprague–Dawley) organs and tissues investigated. Only blood showed no detectable activity, which indicates that the low enzyme activity found in some organs is not due to the presence of blood components in the tissues. In earlier studies with a less sensitive assay, epoxide hydratase activity was detected only in rat liver and kidney but not in organs such as muscle, spleen, heart and brain. Epoxide hydratase was also measured in 6 organs of the mouse (NMRI). The distribution pattern was quantitatively quite different in the two species. The sp. act. in the rat were in the order liver > testis > kidney > lung > intestine ≃ skin. In the mouse, very surprisingly, testis had the highest specific epoxide hydratase activity. Moreover, the order of sp. act. in the mouse organs was remarkably different from that in the rat, namely testis > liver > lung > skin > kidney > intestine. The fact that the sp. act. in kidney was much lower than in lung or skin is most striking. Pretreatment of rats with Aroclor 1254 (a mixture of polychlorinated biphenyls) increased the epoxide hydratase activity in the liver to 175 per cent of the control level. However, the enzyme activity in the 13 extrahepatic tissues investigated was not significantly changed. In organs possessing sufficiently high enzyme levels, epoxide hydratase activity was also measured with styrene oxide as substrate. The ratio of the sp. act. of the two substrates was very similar in rat liver, kidney, lung and testis. This supports the assumption that in these organs a single enzyme is responsible for the hydration of both substrates—as was earlier shown by several methods for the rat liver.

Aromatic and olefinic compounds can be metabolized by mammalian monooxygenases to electrophilically reactive epoxides [1–4], which may covalently bind to cell components [5, 6]. Such epoxides represent prime candidates for the species responsible for the mutagenic and carcinogenic effects of various polycyclic aromatic hydrocarbons [7]. Further biotransformation of these reactive metabolites to the corresponding glutathione conjugates and *trans*-dihydrodiols is catalysed by the cytoplasmic glutathione transferases (EC 4.4.1.7) [8, 9] and by the microsomal epoxide hydratase (EC 4.2.1.63) respectively [1, 2, 10]. The latter enzyme may be especially important not only for the inactivation of ultimately mutagenic epoxides, but also for the formation of dihydrodiols—precursors of the dihydrodiol epoxides, which are possible ultimate carcinogens [11]. Therefore, it is very important to learn which organs possess epoxide hydratase activity.

Hepatic epoxide hydratase has a K_m value in the millimolar range (0.42–0.53 mM) for the alkene oxide, styrene oxide [12, 13]. Strikingly, the K_m value for K-region arene oxides derived from polycyclic hydrocarbons is much lower (2–6 μM) [10, 14], suggesting that the enzyme may have evolved to remove these compounds. If this were the case, epoxide hydratase activity would be expected to be found in all mammalian organs possessing microsomal monooxygenase capable of converting the ubiquitous polycyclic hydrocarbons to their epoxides. However, in earlier studies where a less sensitive assay was used, epoxide hydratase activity was only detected in rat liver and kidney and not in organs such as muscle, spleen, heart and brain [15, 16].

The question whether epoxide hydratase is ubiquitous in the mammalian organism, and whether organs exist whose epoxide hydratase activity is especially high (possibly indicating an endogeneous metabolic function of the enzyme) prompted the present distribution study. Moreover, the ability of Aroclor 1254, a known contaminant of human adipose tissue [17, 18] and human milk [19], to induce epoxide hydratase in the liver and a number of extrahepatic tissues was investigated. The effect of this inducer on epoxide hydratase activity appeared of special interest since it stimulates the biosynthesis of phenobarbital as well as 3-methylcholanthrene inducible monooxygenase activities [20], and because liver preparations from Aroclor 1254-pretreated rats are recommended for use in the activating system in mutagenesis screening [21].

MATERIALS AND METHODS

Chemicals. [^{3}H]benzo[a]pyrene 4,5-oxide was prepared according to the method of Dansette and Jerina [22]. Synthesis, isolation and handling were performed under Argon and exclusion of light. The epoxide was stored at −20° under Argon. The starting material, generally tritiated benzo[a]pyrene (21 Ci/mmole), was obtained from the Radiochemical Centre,

* To whom reprint requests and correspondence should be addressed.

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Amersham, England and diluted to a sp. act. of 1.25 mCi/m-mole. [7-^{3}H]styrene oxide was synthesized as described [15]. Aroclor 1254 (a mixture of polychlorinated biphenyls) was provided generously by Monsanto Company, St. Louis, MO, USA. Other chemicals were of the purest grade commercially available.

Animals. Adult male and female Sprague–Dawley rats (220–280 g) were obtained from Versuchstier-Zuchtanstalt WIGA, Sulzfeld, West Germany. Male NMRI mice (25–35 g) were a gift from Professor E. Pfeiffer, Institut für Hygiene, Universität Mainz.

Preparation of microsomes

General. The animals were killed by cervical dislocation. The organs from groups of 3–5 animals were excised and pooled in ice-cold 1.15% KCl containing 10 mM K phosphate buffer pH 7.4. If necessary, the organs were freed from fat or other adhering tissues under a stereomicroscope. Organs (except for skin, see below) were minced and homogenized in about 3 vol of 1.15% KCl containing 10 mM K phosphate buffer pH 7.4 in a Potter–Elvehjem homogenizer. The homogenates were centrifuged at 10,000 *g* for 15 min and the resulting supernatant fractions were centrifuged at 100,000 *g* for 1 hr. The microsomal pellets were resuspended in the same medium which resulted in final concentrations of 1–5 mg protein/ml for all the tissues except for adrenal gland, fat (kidney), trachea and ovary where lower concentrations (0.25–0.5 mg protein/ml) were obtained.

Skin. After killing, the animals were shaved on the dorsal area (4 × 6 cm) with an electric clipper and the remaining hair was removed using a scalpel. The shaved areas were pooled in ice-cold 1.15% KCl containing 10 mM K phosphate buffer pH 7.4. The following preparations were performed at 4°. The subcutaneous fat tissue (subcutis) and then the deeper pinkish layer (cutis) was removed using a scalpel. The remaining white epidermis was minced. After this separation, it was estimated by morphological examination that cross-contamination of the three skin fractions was less than 10 per cent. The three skin fractions were homogenized separately in about 5 vol. of 1.15% KCl containing 10 mM K phosphate buffer pH 7.4 using an Ultra-turrax for five times 15 sec at high speed. Samples were cooled for 5 min between each Ultra-turrax treatment. A much better yield of microsomal protein was obtained when using 1.15% KCl rather than 0.25 M sucrose. The homogenates were centrifuged as described above.

Protein concentrations were determined by the method of Lowry *et al.* [23] with bovine serum albumin as standard.

Assays. Epoxide hydratase activity was determined using the radiometric extraction assays with [^{3}H]benzo[a]pyrene 4,5-oxide [14] and [7-^{3}H]styrene oxide [15] as substrates. The latter assay was performed under conditions described in [24] (without Tween 80). Enzyme activities are corrected for 80 per cent recovery of benzo[a]pyrene-4,5-dihydrodiol and for 86 per cent recovery of styrene glycol.

RESULTS AND DISCUSSION

Distribution of epoxide hydratase in the rat and mouse. Epoxide hydratase was measured in 25 organs and tissues and in the blood of male as well as in the ovary of female Sprague–Dawley rats using benzo[a]pyrene 4,5-oxide as substrate. Although undetectable in whole blood under the conditions used, enzyme activity was found to be present in all the 26 organs and tissues tested (Table 1). In earlier studies on the distribution of epoxide hydratase in rat organs where styrene oxide was used as substrate, enzyme activity could be detected only in liver and kidney, but not in spleen, brain, heart and muscle [15, 16]. This apparent discrepancy is most probably due to the higher sensitivity of this recently developed assay with benzo[a]pyrene 4,5-oxide as substrate, rather than to a separate enzyme being responsible for the hydration of benzo[a]pyrene 4,5-oxide (see below).

Although the enzyme activity in some organs was very low, it could always be precisely determined. In all cases the activity of the microsomal sample was linear with respect to protein concentration and was at least twice the blank value, except for the preparations from muscle and prostate gland, which resulted in 60 per cent and 70 per cent above blank respectively. The relatively large variations of the sp. act. in fat and adrenal gland is probably due to the exceedingly low yield of microsomal protein in these tissues.

In whole blood containing 0.79% sodium citrate, no activity could be detected, indicating that the low enzyme activity in some organs is not due to blood components present in the tissues. Sodium citrate, at the concentration used, showed no inhibitory effect on epoxide hydratase of rat liver microsomes. Lack of measurable epoxide hydratase activity in whole blood does not, of course, exclude the presence of the enzyme in a minor blood component, such as monocytes or stimulated lymphocytes.

The highest epoxide hydratase activities were found in liver, testis, kidney, ovary and lung. The expectation that organs which come most directly in contact with environmental compounds would, as a consequence of induction by such compounds, or under evolutionary pressure possess especially high epoxide hydratase activities was not confirmed. Thus the sp. act. in skin, trachea, lung, tongue, oesophagus, membranous stomach, glandular stomach, small intestine, caecum and colon were all considerably lower than those of such internal organs as liver, testis and ovary, and of a similar order of magnitude to the remaining tissues. Also, the sp. act. in the three layers of skin, epidermis, cutis and subcutis were not very different from each other (Table 1).

As can be seen from Table 2, in male NMRI-mice the distribution pattern of epoxide hydratase is quantitatively quite different from that in the rat. Most surprisingly, the highest activity was found in the testis which had a sp. act. more than twice as high as the liver. Experiments to elucidate the role of epoxide hydratase in this organ are in progress. In lung and skin the sp. act. were about the same for both species, whereas in liver and kidney of the mice they were 7–10 times lower than in the rat. Thus, the sp. act. of epoxide hydratase in kidney was considerably higher than in the lung and skin of the rat, whereas in the mouse the reverse was true.

Induction by Aroclor 1254. Two groups of 3 male

Table 1. Epoxide hydratase activity in Sprague–Dawley rats measured with benzo[a]pyrene 4,5-oxide as substrate

Organ*	mg protein per assay†	Specific activity‡ Control	Specific activity‡ Aroclor 1254§
Liver	0.07–0.32	6391 ± 636 (n = 8)	11359 ± 708‖ (n = 2)
Testis	0.043–0.28	1472 ± 247 (n = 7)	1046
Kidney	0.09–0.69	705 ± 118 (n = 8)	816 ± 15 (n = 2)
Lung	0.11–1.0	362 ± 46 (n = 10)	378
Adrenal gland	0.036–0.19	196, 322	—
Fat (Kidney)	0.04–0.16	179, 249, 367	—
Bladder	0.1–0.4	90	—
Prostate gland	0.16–0.9	41, 52, 72	—
Trachea	0.045–0.18	166	—
Tongue	0.25–1.0	59	—
Oesophagus	0.15–0.4	87	—
Membranous stomach	0.4–1.48	37, 49	49
Glandular stomach	0.2–2.5	59, 90	67
Small intestine	0.2–1.6	95, 125, 158	134
Caecum	0.33–1.34	80	—
Colon	0.2–1.0	54, 60	56
Epidermis	0.24–1.5	129, 156	144
Cutis	0.2–1.0	57, 66	70
Subcutis	0.1–0.6	82, 115	115
Submaxillary gland	0.15–1.75	240, 293	254
Spleen	0.22–1.46	125, 155	137
Thymus	0.15–0.6	111	—
Brain	0.2–0.8	104	—
Heart	0.4–1.76	20	—
Triceps muscle	0.67–2.68	8, 9	11
Blood		not detectable	not detectable
Ovary	0.024–0.048	556	—

* The organs were taken from male Sprague–Dawley rats (220–280 g) except for the ovary which was taken from a female Sprague–Dawley rat (270 g).

† In this range the assay proceeded linearly with respect to protein concentration.

‡ The sp. act. were determined using microsomes (exception: whole blood) and are given in pmoles benzo[a]pyrene 4,5-dihydrodiol mg protein^{-1} min^{-1}. The values for liver, testis, kidney and lung represent the means of the stated number of experiments (n) ± S.D., where for each experiment the organs of 3–5 animals were pooled and duplicate determinations at 2 different protein concentrations were performed. For the remaining tissues, each given value represents the mean of single determinations at 3 different protein concentrations in an experiment conducted on the pooled organs of 3–5 animals.

§ Two groups of 3 animals received a single dose of 300 mg Aroclor 1254/kg body wt i.p., 5 days before they were killed.

‖ $P \ll 0.001$.

Sprague–Dawley rats (220–250 g) were treated by a single intraperitoneal injection of Aroclor 1254 in sunflower oil (300 mg/kg body wt), 5 days before they were killed. Control animals received no oil, since in many previous experiments oil had no effect upon the epoxide hydratase activity (unpublished observation). The organs of three animals were pooled.

Table 2. Epoxide hydratase activity in male NMRI-mice measured with benzo[a]pyrene 4,5-oxide as substrate

Organ	mg protein per assay†	Specific activity†
Liver	0.04–0.3	901 ± 102 (n = 7)
Testis	0.05–0.2	2186 ± 404 (n = 3)
Lung	0.06–0.5	427 ± 148 (n = 3)
Skin*	0.03–0.9	174 ± 20 (n = 2)
Kidney	0.3–2.0	70 ± 7 (n = 3)
Intestine	0.28–1.1	25

* Subcutis was removed.

† See Table 1.

Table 3. Epoxide hydratase activities with styrene oxide and benzo[a]pyrene 4,5-oxide as substrate in rat liver, testis, kidney and lung

Organ	Epoxide hydratase activities* pmoles Styrene glycol mg protein^{-1} min^{-1}	pmoles BP-4,5-dihydrodiol mg protein^{-1} min^{-1}	Ratio†
Liver	6776 ± 726 (n = 9)	6391 ± 636 (n = 8)	1.06
Testis	1658 ± 254 (n = 3)	1472 ± 253 (n = 7)	1.13
Kidney	755 ± 108 (n = 6)	705 ± 118 (n = 8)	1.07
Lung	456 ± 43 (n = 4)	362 ± 46 (n = 10)	1.26

* Values represent the means of the stated number of experiments (n) ± S.D. For each experiment microsomes from pooled organs of 3–5 animals were prepared and enzyme activities determined in duplicates at 2 different protein concentrations.

† The ratios are calculated from the means of the specific epoxide hydratase activities measured with styrene oxide to that measured with benzo[a]pyrene 4,5-oxide.

Table 4. Comparison of the sensitivity of the epoxide hydratase assays with styrene oxide and benzo[a]pyrene 4,5-oxide as substrate

Substrate	Incubation time min	Product equivalents per assay (nmoles)*		% Above blank
		Blank	Rat skin microsomes	
[^{3}H] Styrene oxide	5	4.01 ± 0.23	4.52 ± 0.32	13
	15	5.89 ± 0.53	7.19 ± 0.44	22
	30	9.14 ± 0.58	11.56 ± 0.55	27
	60	15.35 ± 0.65	19.73 ± 0.69	29
[^{3}H]benzo[a]pyrene-4,5-oxide	5	0.40 ± 0.04	0.78 ± 0.05	95
	15	0.39 ± 0.02	1.60 ± 0.04	310
	30	0.41 ± 0.04	2.75 ± 0.06	570
	60	0.42 ± 0.05	5.04 ± 0.08	1100

* Values represent means of triplicate determinations ± S.D. using boiled (blank) and active rat skin microsomes (0.8 mg protein/assay). In the values shown for the active samples blanks are not subtracted.

As can be seen from Table 1, epoxide hydratase activity in the liver was increased to 175 per cent of the control level, whereas in the extrahepatic tissues investigated the enzyme activity was not significantly altered.

Ratio of epoxide hydratase activities with styrene oxide and benzo[a]pyrene 4,5-oxide in rat liver, kidney, lung and testis. The apparent lack of epoxide hydratase in some organs, when sytrene oxide was used as substrate [15, 16] could be explained by the presence of two different enzymes responsible for the hydration of styrene oxide and benzo[a]pyrene 4,5-oxide. However, when epoxide hydratase activity in liver, kidney, lung and testis of the rat was determined with both substrates, the ratios of the sp. act. of the two substrates were very similar in these four organs (Table 3). This suggests that, at least in these organs, there is only one enzyme responsible for the hydration of the two substrates, unless the two different enzymes were always present in the same ratio in the different organs. This finding agrees with a recent immunoprecipitation study in which it was shown that epoxide hydratase activities in solubilised rat liver microsomes towards styrene oxide and benzo[a]-pyrene 4,5-oxide precipitated simultaneously when titrated with antiserum raised against homogeneous epoxide hydratase [25].

The ratio of epoxide hydratase activity with styrene oxide as substrate as compared to that with benzo[a]-pyrene 4,5-oxide as substrate cannot readily be determined in organs with low epoxide hydratase activity. Although the maximal velocity for the hydration of the two substrates were very similar in all the organs that were investigated (Table 3), the assay with benzo[a]pyrene 4,5-oxide is much more sensitive than that with styrene oxide as substrate. As can be seen from Table 4, this is due to marked differences in the behavior of the blank with respect to incubation time. With styrene oxide the blank after 5 min incubation corresponded to about 4 nmoles styrene glycol and increased, due to the non enzymic hydration, almost linearly up to 15.3 nmoles after 60 min incubation. This relatively high and increasing blank limits the detectability of very low enzyme activities with this assay. In contrast, the blank in the assay with benzo[a]pyrene 4,5-oxide as substrate was 10 times lower after 5 min incubation (i.e. corresponding to 0.4 nmoles product), but even more important, it was found to be independent of the incubation time, which resulted in a 38 times higher sensitivity after 60 min incubation. We therefore believe, that the apparent lack of epoxide hydratase activity in many organs, when measured with styrene oxide as substrate [15, 16], is most probably due only to the lower sensitivity of the assay used in these studies and not to the presence of a different enzyme.

In conclusion, epoxide hydratase was demonstrated to be a very widely distributed enzyme in the rat with a surprisingly high activity in testis. Such activity may be taken to indicate an endogeneous function of this enzyme in the testis or else a protective function against mutagenic epoxides in this germinative gland. Aroclor 1254 was found to induce epoxide hydratase in rat liver, but not in the extrahepatic tissues investigated. This finding may have significance for mutagenicity tests with compounds activated via epoxides when liver microsomes from Aroclor 1254-pretreated rats are used in the activating system as recommended [21].

The ratios of the sp. act. with styrene oxide and benzo[a]pyrene 4,5-oxide as substrates was found to be very similar in liver, lung, kidney and testis of the rat. This is in agreement with the assumption that in all these organs a single enzyme is responsible for the hydration of both substrates.

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