densities by biochemical assay, and calmodulin antibody labeling in the EM has localized the protein on postsynaptic microtubules as well as in the densities (*20*). If the cobaltous ions in the labeled neuron are bound to calcium-binding proteins, the accumulation of cobalt-silver at the synapses would indicate a higher concentration of these proteins there. That the cobalt-silver has been found in four-fifths rather than in all of the synapses is not unprecedented and may reflect on the active state of the labeled synapses at the time of staining. In the crayfish, localization of cobalt-filled synaptic specializations in the light microscope has been correlated with functional synaptic interaction (*21*), but a similar analysis has not been made at the EM level.

This method permits neurons to be physiologically characterized and subsequently stained for EM by iontophoresis. Neuronal processes are readily visible in both the light and the electron microscope. Ultrastructural preservation of the tissue, including the dye-filled cell, is excellent, allowing the study of synaptic contacts onto, as well as from the marked neuron. The aggregation of cobalt-silver at the synaptic membranes facilitates location of the marked synapses in the EM. The accumulation at synapses and on or near microtubules is in good agreement with the sites where calmodulin has been localized.

CHRISTINE E. PHILLIPS*
Department of Zoology,
University of Cambridge,
Cambridge CB2 3EJ, England

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* Present address: Department of Biology, University of Virginia, Charlottesville 22901.

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Cytosolic and Microsomal Epoxide Hydrolases: Differential Properties in Mammalian Liver

Abstract. *The epoxide hydrolase activities of the 100,000*g *pellet (microsomal) and 100,000*g *soluble (cytosolic) fractions of mouse, rat, and guinea pig liver were measured with three closely related compounds used as substrates. Differences between the species in the distribution of the cytosolic and microsomal hydrolases and in their substrate specificities and* p*H optima demonstrate why epoxide hydrolase activity in the cytosolic fraction was not detected earlier in spite of intensive work on the microsomal epoxide hydrolase.*

Many natural and man-made compounds exist in the environment as epoxides, and epoxides may result from biological oxidation of alkenes or arenes. Since some epoxidized compounds are electrophilically reactive and are toxins, mutagens, or carcinogens, it is important to understand their routes of degradative metabolism in mammalian tissue.

In mammals, epoxides are degraded chemically and enzymatically through several pathways. Usually important is their conversion to 1,2-diols by epoxide hydrolases (*1*). These enzymes have been widely assumed to be bound to the endoplasmic reticulum or nuclear membrane (*2*); however, some studies have demonstrated that for many lipophilic substrates (such as terpenoid, steroid, and fatty ester epoxides), most of the epoxide hydrolase activity is present in the 100,000*g* soluble (cytosolic) subcellular fraction (*3, 4*).

We recently found that the cytosolic fraction of mammalian liver also hydrates a wide variety of simple aliphatic epoxides including some known mutagens and carcinogens. Since the substrate selectivity of the cytosolic fraction partially overlaps that of the microsomal fraction, it superficially appears that the results of our studies on the cytosolic epoxide hydrolases contradict those of many studies on the microsomal enzymes. However, the different properties of the cytosolic and microsomal enzymes reported here merely indicate how epoxide hydrolase activity in the cytosolic fraction was overlooked by other laboratories for nearly a decade.

Microsomal epoxide hydrolase is reported to hydrate monosubstituted and *cis*-1,2-disubstituted epoxides (*2, 5*). The cytosolic fraction hydrates the former most rapidly, but it also hydrates *trans*-1,2-disubstituted and tri- and tetrasubstituted epoxides (*3, 4*). Styrene oxide has been used by many researchers for monitoring microsomal epoxide hydrolase activity (*2, 5-10*), but minimal hydration of styrene oxide was detected in the cytosolic fraction under several conditions employed in our study (*11*). Two closely related compounds, which differ from styrene oxide by a single carbon atom (Fig. 1), are rapidly hydrated (Table 1) (*12, 13*). These compounds include *trans*-β-methylstyrene oxide, which is hydrated by the cytosolic but not the microsomal fraction, and allyl-

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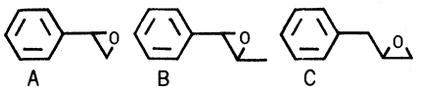

Fig. 1. Structure of the three substrates. (A) Styrene oxide. (B) *trans*-β-Methylstyrene oxide. (C) Allylbenzene oxide.

benzene oxide, which is rapidly hydrated by both fractions. The enzyme activity data given in Table 1 were obtained with the *p*H of the cytosolic and microsomal fractions held at 7.4 and 9.0, respectively. These are the *p*H optima for the substrates examined; Fig. 2 illustrates that very little cytosolic epoxide hydrolase activity is detected when assays are performed at a high *p*H.

The activity levels of cytosolic and microsomal epoxide hydrolase on allylbenzene oxide were substantially different among the three rodent species (Table 1). In the mouse, activity was low in the microsomal fraction but high in the cytosolic fraction; in the guinea pig, activity was moderate in the cytosolic fraction but very high in the microsomal fraction. Cytosolic epoxide hydrolase activity was lowest in the rat, yet this animal has been used for many studies on the subcellular distribution of epoxide hydrolases (*2, 4, 6, 8, 14, 15*).

Although reference is often made in the literature to microsomal epoxide hydrolases, subcellular enzyme distribution studies are surprisingly few in number, and two actually report some hydrolase activity in the cytosolic fraction (*15, 16*). In 1968, Jerina *et al.* (*16*) reported that benzene oxide is hydrated largely, but not exclusively, in the microsomal fraction. In that study, enzyme assays were performed at *p*H 8.0—in part to stabilize the substrate. As shown in Fig. 2, a high *p*H may substantially decrease the amount of cytosolic hydrolase activity observed. Maynert *et al.* (*15*) reported an elegant study on the hydration of three aliphatic epoxides in the rat in which ~ 85 percent of the hydrolytic activity in the postmitochondrial supernatant was microsomal, with the rest cytosolic. The nonmicrosomal activity was incorrectly considered to be an artifact. Had they used the mouse, guinea pig, or a variety of other mammals, they probably would have found very high cytosolic activity since such aliphatic epoxides are rapidly hydrated by the cytosolic fraction.

Styrene oxide, in spite of some difficulties with its volatility and stability, is a good model substrate for microsomal epoxide hydration. It appears to be hydrated by the same microsomal enzyme that hydrates some arene oxides (*8, 17*). Studies of its hydration led to the conclusion that epoxide hydrolase activity is exclusively membrane-bound (*2, 5, 8*). The apparent thoroughness of these studies resulted in the general acceptance of styrene oxide as a model substrate for epoxide hydrolase activity and of the doctrine of membrane-bound epoxide hydration. With a few exceptions (*11, 14, 18*), subsequent studies of subcellular distribution of this enzyme relied on the hydration of styrene oxide in rat tissue at a high *p*H. These three conditions minimized the likelihood that cytosolic epoxide hydrolase activity would be observed and helped to reaffirm the belief that epoxide hydrolase activity is exclusively membrane-bound.

Thus the results of studies by this laboratory are not in conflict with the data presented in most reports on epoxide hydrolases. The conception that epoxide hydrolase activity is not present in the cytosolic fraction resulted from a slight overinterpretation of the data from a few well-designed experiments. Had those experiments been performed in a different order with other model substrates, it might have been concluded that epoxide hydrolase activity is an exclusively cytosolic phenomenon. This report illustrates the importance of investigating the metabolism of new substrates under a variety of incubation conditions. Researchers should recognize that there is extramicrosomal epoxide hydration and begin to evaluate its role in biochemical pathways and xenobiotic metabolism (*19*).

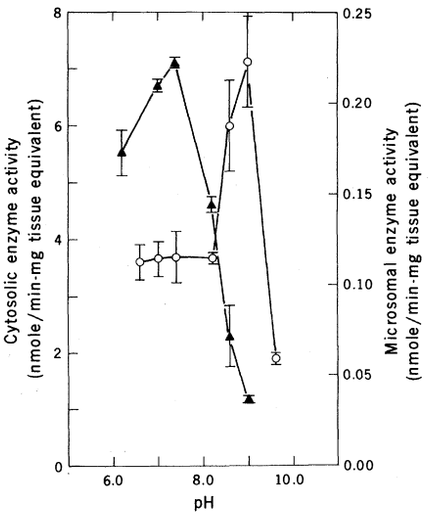

Fig. 2. Activities of the cytosolic (▲) and microsomal (○) epoxide hydrolases from mouse livers on allylbenzene oxide at different *p*H's. Each point represents the mean value for three samples (± standard error) and has been corrected for spontaneous hydration. Samples were assayed by incubating 10 μmole of allylbenzene oxide for 20 minutes at 37°C in 1 ml of enzyme preparation that had been incubated for 5 minutes (*12, 13*). The *p*H activity profile for styrene oxide in microsomes is broader than that for allylbenzene oxide, but the optimum *p*H is also 9 (*2*).

Table 1. Epoxide hydrolase activity of cytosolic and microsomal fractions on three substrates.

Species	Subcellular fraction	Protein* (μg/ml)	Epoxide hydration rate (pmole/min per milligram of tissue equivalent)		
			Styrene oxide	*trans*-β-Methylstyrene oxide	Allylbenzene oxide
Mouse	Cytosolic	59	N.D.†	1890 ± 150	7000 ± 60
	Microsomal	97	85 ± 49	N.D.	190 ± 20
Rat	Cytosolic	43	N.D.	167 ± 110	1570 ± 100
	Microsomal	214	124 ± 4	N.D.	350 ± 36
Guinea pig	Cytosolic	57	N.D.	490 ± 20	2640 ± 115
	Microsomal	161	432 ± 13	N.D.	3600 ± 240

*Protein content was determined by a modification of the Lowry method, with bovine serum albumin used as the standard. The data given in this table were obtained by assaying the microsomal and cytosolic fractions at volumes equivalent to 1 and 0.1 percent of the wet weight of the original liver homogenate, respectively. †Rates less than 3 pmole/min per milligram of tissue equivalent are listed as not detected (N.D.). In $5 \times 10^{-5} M$ styrene oxide, enzyme hydration rates up to only 20 pmole/min per milligram were detected for the mouse cytosolic fraction. This apparent inhibition by styrene oxide is not unexpected, since it has been observed in human liver microsomes (*20*).

KENJI OTA
BRUCE D. HAMMOCK
Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside 92521

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12. Liver homogenates were prepared from male Swiss-Webster mice (12 weeks old, 35 to 45 g), Sprague-Dawley rats (250 to 500 g), and Hartley guinea pigs (500 to 600 g). The livers were freed of adhering tissues, perfused with sodium phosphate buffer (*p*H 7.4, ionic strength, 0.2), and homogenized (10 percent, weight to volume) in the same buffer. Differential centrifugation (*2, 3*) produced subcellular fractions. Washed microsomes were resuspended in tris-HCl (*p*H 9.0, ionic strength, 0.1) and assayed within 48 hours of preparation. Cytosolic fractions were diluted with tris-HCl (*p*H 7.4, ionic strength, 0.2). The repelleting procedure often results in a substantial decrease in *trans*-β-methylstyrene oxide hydrolase activity in the unwashed microsomal fraction; the > 96 percent recovery of styrene oxide epoxide hydrolase activity after repelleting indicates that the loss in microsomal activity is due to the removal of the trapped cytosolic fraction.
13. Epoxide hydrolase activity was monitored by gas-liquid chromatography of the *n*-butylboronate diesters of the diols. Homologous diols were used as internal standards. Rates were determined from three replicates of at least four points on a linear time course, and rates of hydration were proportional to the time of incubation and to the protein concentration. The microsomal enzyme activity for which styrene oxide was used as a substrate is similar to that demonstrated elsewhere (*9, 10*).
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X-ray and Neutron Scattering Density Profiles of the Intact Human Red Blood Cell Membrane

Abstract. *Hemoglobin-free human red blood cell membranes have been prepared with glutaraldehyde to maintain an intact structure on partial dehydration. Treatment of resealed ghosts with poly(L-lysine) produced an essentially constant structural unit and permitted correlation of electron microscopy results with x-ray and neutron diffraction profiles. These profiles provide detailed information, for the intact membrane, on the location and relative distribution of lipids and proteins.*

The red cell membrane has been the object of structural investigations for many years. Although its major components have been characterized, and to some extent biochemically localized, their physical distribution is still largely unknown. Until now, no one membrane specimen has been prepared for x-ray diffraction which is sufficiently well organized for reasonable resolution and produces an electron density profile (*1-3*) that is compatible with biochemical results (*4*). We report here the determination of an electron density profile from intact hemoglobin-free erythrocyte membranes that is consistent with biochemical results and compatible with electron micrographs of such membranes. We also present neutron scattering profiles for this system.

In this work we used standard hemoglobin-free Dodge membranes (*5*) that were subsequently resealed (*6*). Removal of water from a suspension of ghosts by equilibrating the pellet over a saturated salt solution has been used to organize membranes on a one-dimensional lattice (*2, 7*). We found that the red cell membrane does not remain intact under these conditions (*8*); at least one major protein component, spectrin (which resides at the cytoplasmic surface), is displaced during the partial dehydration, giving a residual membrane specimen that produces an x-ray periodicity of 55 to 70 Å. Treatment of the membrane suspension with 1 percent glutaraldehyde before partial dehydration eliminated this repeat and produced three to four orders of a 200- to 300-Å periodicity (*9*). As estimated by dry weight, the glutaraldehyde produced less than a 5 percent increase in the membrane mass. Electron micrographs of partially dehydrated membranes fixed in glutaraldehyde and OsO_4 and examined edge-on showed a highly asymmetric pair of collapsed membranes (each membrane about 120 to 150 Å thick) with variable spacing between adjacent membrane pairs (Fig. 1a). Resolution was sufficiently good that we could identify an unstained ribbon ~25 Å thick (presumably the hydrophobic core of a lipid bilayer) bordered by a thin (~25 Å) band of stained material at the extracellular face and a thick stained region (~70 to 100 Å) on the cytoplasmic side.

In order to establish, for the purpose of phasing, a structural unit that remains constant at different lattice spacings and is readily identifiable in electron micrographs, resealed ghosts were cross-bridged at their extracellular surfaces with poly(L-lysine) (producing a mass increase of no more than 1 percent). Poly(L-lysine) is known to aggregate whole cells and ghosts by interacting with the net negative charge on the exte-

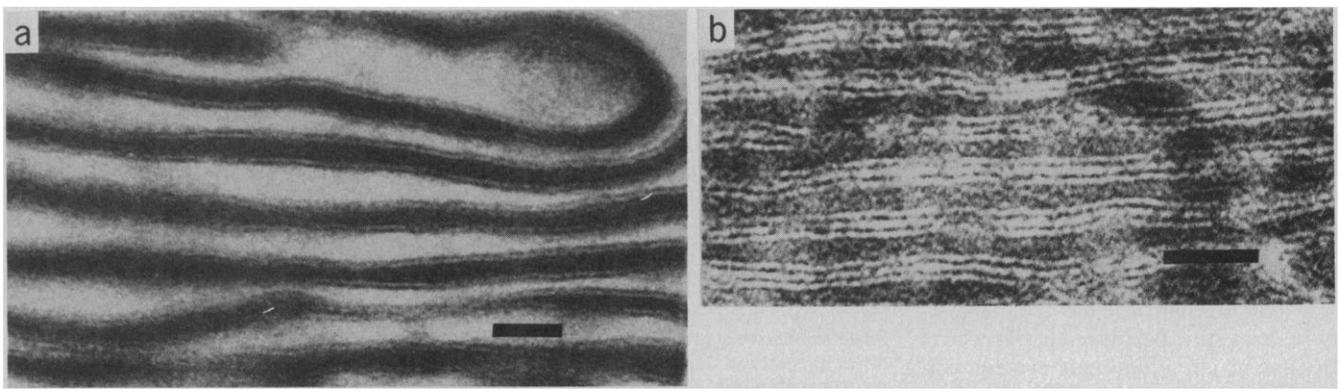

Fig. 1. Electron micrographs of resealed, glutaraldehyde-fixed, and partially dehydrated human red cell ghosts (a) without poly(L-lysine) and (b) treated with poly(L-lysine). Scale bars, 50 nm. In (b) the unit consisting of three thin unstained ribbons corresponds to the extracellular space between membrane pairs and the hydrocarbon cores of the membrane lipid bilayers on either side.

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