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DISTRIBUTION OF POLYCYCLIC HYDROCARBON METABOLISM-LINKED ENZYMES IN SPECIALIZED REGIONS OF THE ENDOPLASMIC RETICULUM

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Summary

Microsomal enzymes were partially separated by centrifugation in continuous sucrose gradients containing a low concentration of detergent into four bands in which the following enzymes were enriched: (I) glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9); (II) cytochrome *P*-450, NADPH—cytochrome *c* reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4), monooxygenase (7-ethoxycoumarin-*O*-deethylase); (III) cytochrome b_5, NADH cytochrome *c* reductase (NADH:ferricytochrome oxidoreductase, EC 1.6.2.2), epoxide hydratase (glycol hydro-lyase (epoxide-forming), EC 4.2.1.63), and UDPglucuronyltransferase (UDPglucuronate β-glucuronosyltransferase (acceptor-unspecific), EC 2.4.1.17); (IV) glutathione-*S*-transferases (RX:glutathione R-transferase, EC 2.5.1.18). The distribution of epoxide hydratase in the active fractions was identical whether determined with styrene oxide or benzo(a)pyrene-4,5-oxide as substrate giving further support to the conclusion that both substrates are hydrated by the same enzyme. The fact that epoxide hydratase, UDPglucuronyltransferase and microsomal glutathione-*S*-transferase activities were not enriched in the same fractions as the cytochrome *P*-450 monooxygenase system argues against a firm spatial association of the bulk of any of the former enzymes with the bulk of the monooxygenases system, although they catalyze consecutive reactions. Inclusion of antibodies to epoxide hydratase in the sucrose gradients inhibited the epoxide hydratase activity but did not affect monooxygenase activity which again suggests spatial independence of these enzymes.

Introduction

The spatial relationship of the enzymes in the endiplasmic reticulum is of general interest because these enzymes catalyse consecutive reactions of foreign compounds. The monooxygenase system produces both epoxides and phenols [1—8]. The phenols may be conjugated with glucuronic acid, a reaction catalysed by UDPglucuronyltransferase [9]. The epoxides are further metabolized by enzymes catalysing the attack of nucleophilic agents and may be converted to *trans*-dihydrodiols through the action of epoxide hydratase (epoxide hydrase) [2,3] or conjugated with glutathione, a reaction catalyzed by the glutathione-*S*-transferases [10,11] which, although found mainly in the cytoplasm, are also associated with the microsomal membrane [12,13]. Attempts have been made to detect structures within the endoplasmic reticulum whose enzymes form functional units like those in the mitochondrial inner membrane which are concerned with the oxidative phosphorylation [14].

Oesch and Daly [15] found when studying the conversion of naphthalene to 1,2-dihydro-1,2-dihydroxy-naphthalene that, although naphthalene oxide is an obligatory intermediate, externally added naphthalene oxide did not freely exchange with that formed from naphthalene. To explain these results the authors suggested a close spatial relationship between monooxygenase and epoxide hydratase within the microsomal membrane. This suggestion is particulary interesting in view of the relationship between these two enzymes in the metabolism of polycyclic hydrocarbons to mutagens [16,17] and thus requires further investigation.

Recently Winqvist and Dallner [14] have shown that the NADPH- and NADH-dependent electron transport systems of the rat liver endoplasmic reticulum can be partially separated by density gradient centrifugation after treatment of microsomes with detergent concentrations lower than those required to dissolve the membranes. This system allows simple investigation of possible interrelationship between microsomal enzymes.

We report here the distribution in continuous sucrose gradients of several enzymes located in the microsomal membrane, most of which are concerned with the metabolism of carcinogenic polycyclic hydrocarbons, using a modification of the method described by Winqvist and Dallner [14].

Methods

Tissue preparation. Livers were taken from male Sprague-Dawley rats (200—220 g). Livers from three rats were combined and microsomal fractions were prepared as described by Dallner [18]. Microsomal fractions so prepared were washed twice by resuspending in 0.25 M sucrose in 10 mM Tris-HCl buffer (pH 7.6)/50 mM KCl and centrifuged at 100 000 $\times g_{max}$ for 1 h. Such treatment removed cytoplasmic glutathione-*S*-transferases [13] and was therefore essential to determine the microsome-associated glutathione-*S*-transferases activity.

Continuous gradient centrifugation. 8.0 g sucrose for the lighter solution or 61.5 g for the heavier solution were dissolved with 0.2 g sodium deoxycholate, 10 ml glycerol, and 5 mmol KCl in 10 mM Tris-HCl (pH 7.6) to give a final volume of 100 ml. The gradient thus covered the density range of $d = 1.03$—

1.21 g/ml. When cytochrome *P*-450 content and monooxygenase (7-ethoxycoumarin-*O*-deethylase) activity were determined, all solutions and the gradient contained 10^{-4} M dithioerythritol. The washed microsomal fractions were resuspended in the light solution to give a protein concentration of 6 mg/ml. 3 ml of this suspension was layered on top of the preprepared gradients. Centrifugation was performed at 110 000 × g_{max} in a Beckman SW-27 rotor for 40 h at 0—3°C. After centrifugation the gradients were fractionated into 30 aliquots which were assayed for the relevant enzyme activities.

Enzyme assays. Epoxide hydratase (glycol hydro-lyase (epoxide-forming), EC 4.2.1.63) activity was measured using [^{14}C]styrene oxide [19] and [^{3}H]-benzo(a)pyrene-4,5-oxide [20] as substrates. Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) was assayed as described by Aronson and Touster [21], measuring inorganic phosphate as a phosphomolybdate complex [22]. Monooxygenase activity was estimated using the O-deethylation of 7-ethoxycoumarin [23]. UDPglucuronyltransferase (UDP-glucoronate β-glucuronosyltransferase (acceptor-unspecific), EC 2.4.1.17) activity was measured using 1-naphthol as aglycone substrate [24] and glutathione-*S*-transferase (RX:glutathione R-transferase, EC 2.5.1.18) activity was estimated using 1-chloro-2,4-dinitrobenzene as substrate [25]. The cytochrome b_5, cytochrome *P*-450, and cytochrome *P*-420 content was measured as described by Omura and Sato [26] and the NADH- (NADH:ferricytochrome oxidoreductase, EC 1.6.2.2) and NADPH- (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4)-dependent cytochrome *c* reductase activities were measured as standard.

Treatment with antibodies against epoxide hydratase. Antiserum against homogenous epoxide hydratase [30] was raised in rabbits as described [31]. An IgG fraction was prepared by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography of the antiserum. Sucrose gradients were additionally loaded with 0.7 ml IgG fraction (22 mg protein/ml) to test the inhibition of epoxide hydratase by its antibody.

Results and Discussion

Distribution in the sucrose gradient

Visual inspection of the sucrose gradients after centrifugation of the rat liver microsomes resuspended in low concentrations of detergent showed several bands which were detectable because of the light scattering. However, it seemed to us more practical to fractionate the whole gradients than to attempt to remove the individual bands since this allowed us to express the enzyme activities as units/ml rather than as relative specific activities. This is important because an uneven distribution of the cell components throughout the gradient may lead to shifts in the position of maxima when expressed as relative specific activities.

Fig. 1 shows the distribution of the measured parameters within the density gradients. Although routinely four light-scattering bands were visible only two protein peaks were observed, a small peak at d = 1.14 g/ml and a larger peak at d = 1.06 g/ml. The RNA was found to be solely associated with the smaller protein peak at d = 1.14 g/ml which also contained the majority of the glucose-6-phosphatase activity. All the other enzyme activities measured were asso-

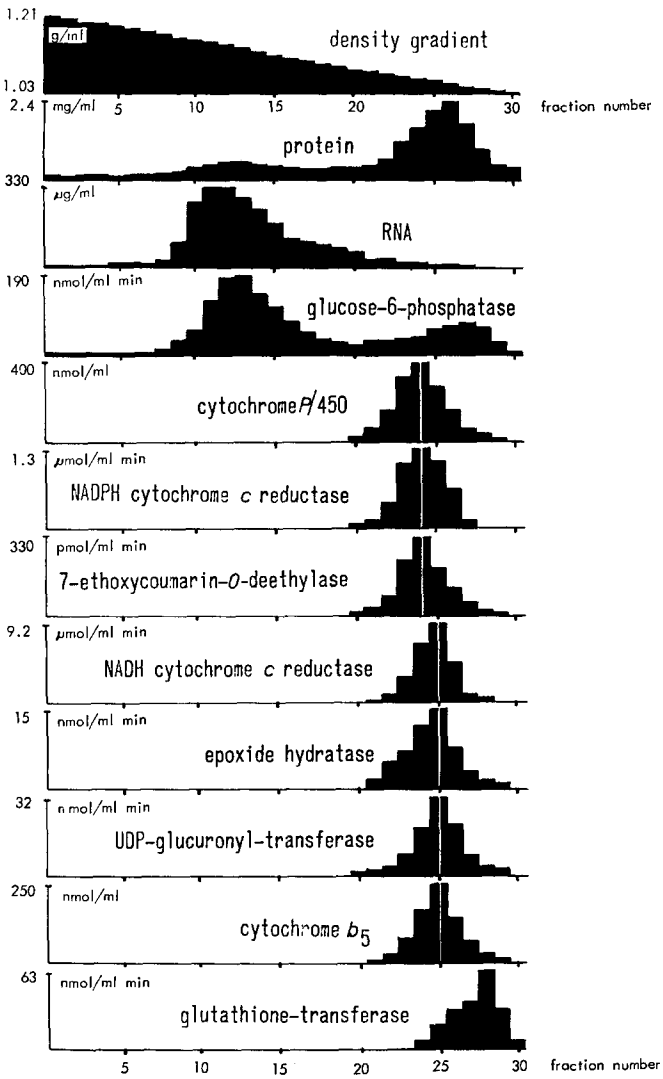

Fig. 1. Distribution of microsomal enzyme activities, protein and RNA in deoxycholate-containing sucrose density gradients. The distribution of protein, RNA, and enzymes of the endoplasmic reticulum which are either characteristic for this compartment or concerned with the metabolism of polycyclic aromatic hydrocarbons was analyzed in a sucrose gradient containing 0.2% sodium deoxycholate and 10% glycerol (run time 40 h, 110 000 × g_{max}). According to their distribution the enzymes were grouped as follows: group I, glucose-6-phosphatase; group II, cytochrome *P*-450, NADPH cytochrome *c* reductase, monooxygenase (7-ethoxycoumarin-*O*-deethylase); group III, epoxide hydratase (substrate: benzpyrene-4,5-oxide), UDPglucuronyltransferase, NADH cytochrome *c* reductase, cytochrome b_5; group IV, glutathione transferase.

ciated with the protein peak in the less dense region of the gradient. The remainder of the glucose 6-phosphatase activity was found in fractions of the gradient with a lower density than those in which most of the other enzymes were enriched. The microsome-associated glutathione transferase activity was located near the top of the gradient. The maximum activity was well separated from those of the other microsomal enzymes.

As observed by Winqvist and Dallner [14] there was a partial separation of the NADPH cytochrome *c* reductase from the NADH cytochrome *c* reductase. The former had its maximum activity in more dense fractions than the latter. The monooxygenase activity (substrate: 7-ethoxycoumarin) was easily measurable even after 40 h centrifugation and showed a distribution similar to that of NADPH cytochrome *c* reductase. The maximum content of cytochrome *P*-450 was found in the same fractions.

The epoxide hydratase activity had the same distribution throughout the gradients when measured either with styrene oxide or benzo(a)pyrene oxide as substrates and the ratio of the hydration rates of these two substrates was similar in all active fractions (Fig. 2). This supports the conclusion of Oesch and Bentley [31] that these two substrates are hydrolysed by the same enzyme in rat liver microsomes. The distribution of epoxide hydratase was similar to that of the NADH-dependent cytochrome *c* reductase and cytochrome b_5, the highest activities were found in fractions of lower density than those containing NADPH cytochrome *c* reductase, cytochrome *P*-450 and 7-ethoxycoumarin-*O*-deethylase. The UDPglucuronyltransferase activity measured with 1-naphthol as substrate was active despite the length required for the centrifugation and the presence of deoxycholate at concentrations which inhibit the conjugation of *p*-nitrophenol [32]. The distribution of this enzyme was also very similar to that of NADH cytochrome *c* reductase, and cytochrome b_5.

Consequently with the exception of glucose-6-phosphatase and glutathione-*S*-transferases the enzyme activities studied separated into two groups, one group containing the NADPH cytochrome *c* reductase, cytochrome *P*-450 and 7-ethoxycoumarin-*O*-deethylase and the second group containing NADH cytochrome *c* reductase, cytochrome b_5, epoxide hydratase, and UDPglucuronyltransferase. Although the separation of these groups of enzymes was only partial, the results were very reproducible. It is of interest that the enzymes which catalyse the further metabolism of products of the monooxygenase reaction are not enriched in the same fractions as the monooxygenase activity.

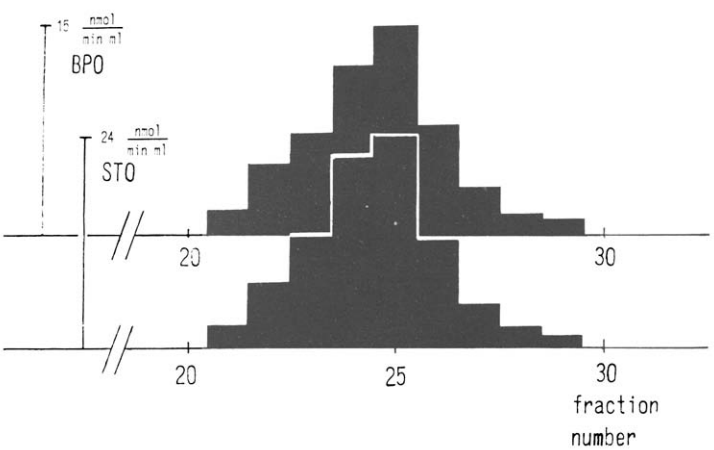

Fig. 2. Distribution of epoxide hydratase in deoxycholate-containing sucrose gradients. Sucrose gradient fractions were assayed for epoxide hydratase activity with [^{3}H]benzpyrene-4,5-oxide (BPO) and [^{14}C]-styrene oxide (STO) as substrates.

Centrifugation in stretched sucrose gradients

Attempts were made to increase the separation between the two groups of enzymes by stretching the lighter part of the gradient using a density range similar to the upper half of the standard gradients and increasing the centrifugation time from 40 to 60 h. Such alteration did not lead to total separation of the two enzyme groups, but did result in a greater physical separation of the fractions with maximum activities of the measured enzymes (Fig. 3). Again epoxide hydratase and UDPglucuronyltransferase showed a distribution similar to that of NADH cytochrome *c* reductase whereas the 7-ethoxycoumarin-*O*-deethylase activity was enriched in the same fractions as NADPH cytochrome *c* reductase.

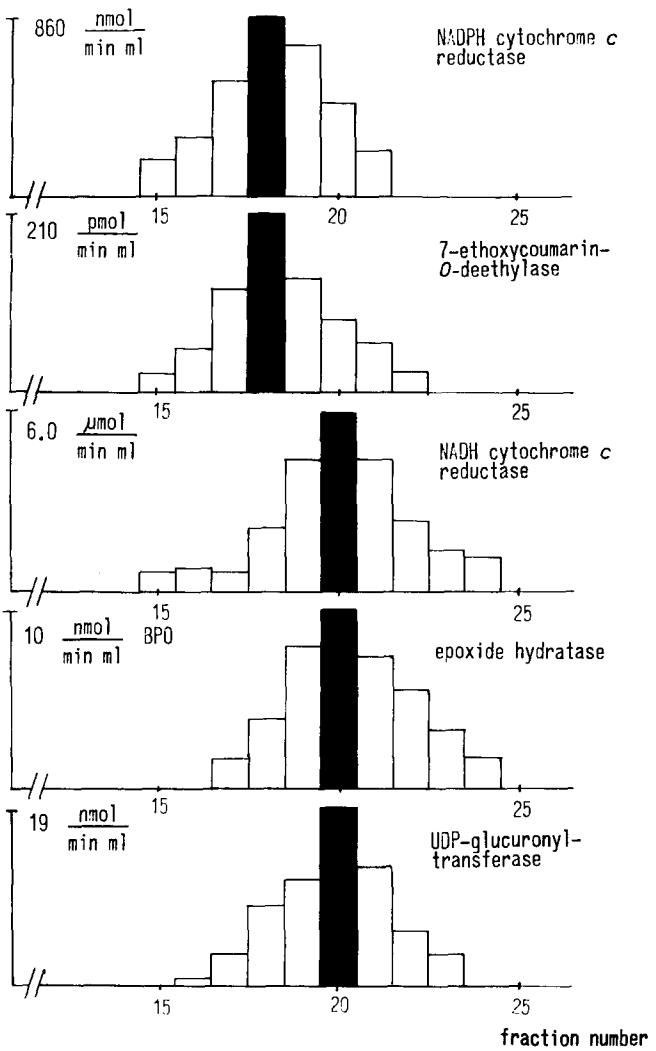

Fig. 3. Distribution of enzyme activites in stretched gradient. The lighter part of the gradient was stretched by using gradients with a density range corresponding to the upper half of the standard gradients. The run time of the centrifuge was increased from 40 h to 60 h.

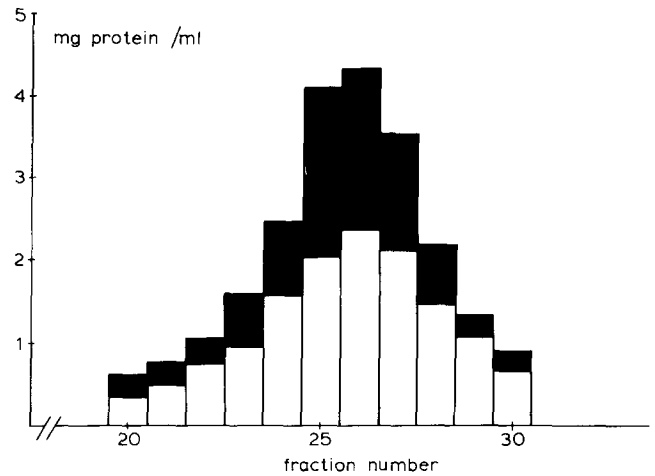

Fig. 4. Effect of addition of antibodies (IgG fraction) against epoxide hydratase to the sucrose gradients. Graph showing the distribution of protein in the upper part of the gradient with antibodies against epoxide hydratase (filled bars + empty bars) and without antibodies (empty bars). Treatment of the microsomes with IgG antibodies against epoxide hydratase results in a decrease of activity of this enzyme to practically zero. The activity of the monooxygenase system was thereby unaffected. Separation conditions are described in Fig. 1.

Examination of the protein-containing fractions using electron microscopy showed that closed membrane vesicles were still present in the fractions. This suggests that the separation of the two groups of enzyme activities was not simply the result of aggregation of solubilized proteins but the result of separation of microsomal vesicles with different protein compositions. In this context it is particularly interesting that epoxide hydratase is not concentrated in the same fractions as the monooxygenase and, although the results do not show a total separation into vesicles which contain epoxide hydratase and those which contain the monooxygenase system, they strongly suggest that the bulk of the epoxide hydratase is not associated with the monooxygenase in the microsomal membrane. The same argument also applies to the other investigated phase II enzymes.

Treatment with antibodies against the epoxide hydratase

When an anti-epoxide hydratase IgG fraction was included in the sucrose gradient the epoxide hydratase activity was reduced to practically zero. The binding of the antibody could be detected by the high increase of the protein concentration at the loci of the gradient where the epoxide hydratase activity otherwise occurred (Fig. 4). The activity of the monooxygenase system was not affected by the antibody. If a strong spatial relationship between these two systems existed, a reduction of the monooxygenase activity through steric hindrance by the antibody may be expected. Our experiments contradict this hypothesis.

Conclusion

The results of these studies show that vesicular structures are present in microsomal fractions treated with low detergent concentration and that these

vesicles may be separated according to their density in sucrose gradients. This results in a partial separation of the NADH- and NADPH-dependent electron transport systems as shown previously by Winqvist and Dallner [14] and of the cytochrome *P*-450 dependent monooxygenase from the enzymes responsible for the further metabolism of the products of the former. The results suggest that a firm spatial association between the cytochrome *P*-450 monooxygenase system and epoxide hydratase or between the cytochrome *P*-450 monooxygenase system and UDPglucuronyltransferase measured with 1-naphthol as substrate are unlikely.

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