*Biochemical Pharmacology*, Vol. 37, No. 6, pp. 1103–1109, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

# A NOVEL BIOLOGICALLY ACTIVE SELENOOORGANIC COMPOUND—VII

## BIOTRANSFORMATION OF EBSELEN IN PERFUSED RAT LIVER

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(*Received* 19 *May* 1987; *accepted* 17 *September* 1987)

**Abstract**—Ebselen (PZ 51) is a selenoorganic compound with antioxidant and antiinflammatory properties, and its metabolism was studied in isolated perfused rat liver (hemoglobin-free, open system). $^{75}$Se-labelled ebselen was taken up into liver cells and radioactivity was excreted into bile. Biliary excretion of $^{75}$Se-compounds reached maximal values of 4 nmol/min per g wet wt.

HPLC analysis of bile and effluent perfusate as well as identification of separated metabolites by mass spectrometry were carried out. The biliary metabolites were (a) an interesting novel Se-glucuronide, 2-glucuronylselenobenzanilide, (metabolite IV), as the major metabolite, and (b) an *O*-glucuronide, *N*-(4′-glucuronyloxyphenyl)-2-methylselenobenzanilide (metabolite III). The major effluent perfusate metabolites were Se-methylated derivatives (metabolites I and II).

There was no evidence for sulfated metabolites. The selenodisulfide with glutathione, *S*-(2-phenylcarbamoyl-phenylselenyl)-glutathione, was not detected, probably because of low steady-state concentrations and/or its biochemical lability.

The selenium in ebselen is not bioavailable (e.g. for the synthesis of glutathione peroxidase), in contrast to selenite, for example, thus explaining the very low ebselen toxicity. However, the enzymatic steps in Se-methylation could be similar to those in the metabolism of selenite which include hydrogen selenide methylation. Se-glucuronides constitute a novel category of compounds in addition to the *O*-, *N*-, *C*- and *S*-glucuronide classes known in biology.

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, PZ 51) is an organoselenium compound which exhibits a glutathione peroxidase (GSH-Px)-like activity by catalyzing the reduction of $H_2O_2$ and other hydroperoxides *in vitro* [1, 2]. In addition, a glutathione-independent antioxidant capacity was found during microsomal lipid peroxidation [1], similar to that observed with diethyldithiocarbamate [3]. Ebselen was also able to inhibit ADP-Fe-induced lipid peroxidation in isolated hepatocytes, dependent on the presence of intracellular glutathione [4]. In the isolated perfused rat liver, ebselen was capable of diminishing ethanol-induced ethane release, whereas a sulfur analog was not [5]. Incubation of resident mouse macrophages with ebselen revealed a partial inhibition of prostaglandin $E_2$ production, implying a hydroperoxide scavenging GSH Px-like activity of this compound [6]. Recently, it was found that ebselen decreased significantly the cytotoxicity of quinones in Ehrlich ascites cells, an effect attributable to the antioxidant properties of ebselen [7]. Furthermore, anti-inflammatory effects of ebselen were demonstrated [8, 9], and it was suggested that this effect is attributable to an inhibition of lipoxygenase and/or an isomerisation of lipoxygenase products by ebselen [9–11].

Here we present observations on the biotransformation of ebselen in the isolated perfused rat liver, with the demonstration of a novel type of glucuronide, an Se-glucuronide. Metabolic studies with intact animals and the detection of metabolites in plasma and urine are reported elsewhere in Ref. 12.

### MATERIALS AND METHODS

Livers from male Wistar rats (180–220 g body weight), fed on stock diet (Altromin, Lage, F.R.G.) were perfused at 37° without recirculation of the perfusate, using bicarbonate-buffered salt solution equilibrated with $O_2/CO_2$ (19:1, v/v) as described [13]. The perfusate was supplemented with taurocholic acid (5 $\mu$M). Perfusate flow (4–5 ml/min per g wet wt) was maintained constant throughout the individual experiment. The concentration of $O_2$ and the pH in the effluent perfusate were monitored using appropriate electrodes and care was taken to maintain effluent $O_2$ concentration above 0.2 mM to avoid pericentral hypoxia. $^{75}$Se-labelled compounds were quantitated using a Multi-Prias gamma counter (Packard Co.).

The concentration of ebselen in the portal perfusate was checked photometrically against a standard solution at 313 nm. Metabolites of ebselen were analyzed in the effluent perfusate and bile. The effluent (1280 ml) and the bile (400 $\mu$l) were collected over a time period of 40 min. Portions of 300 ml of the effluent and half of the collected bile were used

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for subsequent extraction. The organoselenium compounds were extracted twice both with ethyl acetate and dichloromethane, followed by evaporation of the solvents. The combined residue was dissolved in DMSO (0.5 ml or 1.0 ml) and after centrifugation stored for HPLC analysis. Recovery was >90% with this extraction procedure. In some preparations, the residue was incubated with beta-glucuronidase (in 75 mM phosphate buffer, pH 6.8) or sulfatase (in 0.1 M acetate buffer, pH 5) at 37° for 3 hr, followed by extraction and preparation for HPLC as described above. Samples of 50 μl were injected to the reverse phase column (RP18, 100 × 8 mm, LiChrosorb 10 μm; Waters Associates). Separation was performed using a 0.1% $H_3PO_4$/acetonitrile gradient, at a flow rate of 1.5 ml/min. The gradient was from 70/30 to 3/97 in four 10 and 5 min intervals. The metabolites were measured at 313 nm. The synthesis and spectral characteristics of the metabolites is described in Ref. 14.

Mass spectrometric analysis of the metabolites was carried out with a MAT 311 A mass spectrometer (Finnigan, Bremen, F.R.G.). Electron energy was 70 eV, xenon was used with a FAB gun operated with 6–6.5 kV (Iontech, Teddington, U.K.). An ion current of 12–15 μA (ionized xenon) was obtained at an ion source pressure of $5 \times 10^{-4}$ Torr. Fractions of the HPLC eluate were collected, evaporated to dryness under reduced pressure and introduced directly into the mass spectrometer by a solid probe head.

Chemicals and biochemicals were obtained from Merck (Darmstadt, F.R.G.) and Boehringer (Mannheim, F.R.G.). Beta-glucuronidase from *E. coli*, type VII (EC 3.2.1.31), and sulfatase from limpets, type V (EC 3.1.6.1.), were obtained from Sigma (Munich, F.R.G.). Ebselen and analogues were from Nattermann (Cologne, F.R.G.). $^{75}$Se-ebselen (specific activity 16 μCi/mmol) was a gift from Dr Cantineau (University of Liège, Belgium) [15].

## RESULTS

### *Distribution of $^{75}$Se-labelled compounds in liver, bile and effluent perfusate*

Perfusion of the isolated rat liver with different concentrations of $^{75}$Se-ebselen led to a release of $^{75}$Se-compounds into bile and effluent (Fig. 1). This release reached a maximum in the bile after 25–30 min of infusion of $^{75}$Se-ebselen. When the infused concentration was increased from 6.2 to 11.1 μM $^{75}$Se-ebselen, no significant change of the release into the bile was obtained, indicating a maximal release of approximately 4 nmol $^{75}$Se-compounds/min per g liver wet weight. With the infusion of 2.3 μM $^{75}$Se-ebselen, the maximal biliary release was about 25% lower than with 6.2 or 11.1 μM. In contrast, the release of $^{75}$Se-compounds into the effluent perfusate rose proportionally to the infused $^{75}$Se-ebselen concentration (Fig. 1). Table 1 summarizes the distribution of $^{75}$Se-compounds in liver, bile and effluent. It appears that the uptake of $^{75}$Se-ebselen by the liver (or its storage capacity) for $^{75}$Se-compounds is limited. This is indicated by only a minor rise of the $^{75}$Se-content in the liver after elevating the amount of $^{75}$Se-ebselen in the influent fron 8.6 to 15.5 μmol (Table 1).

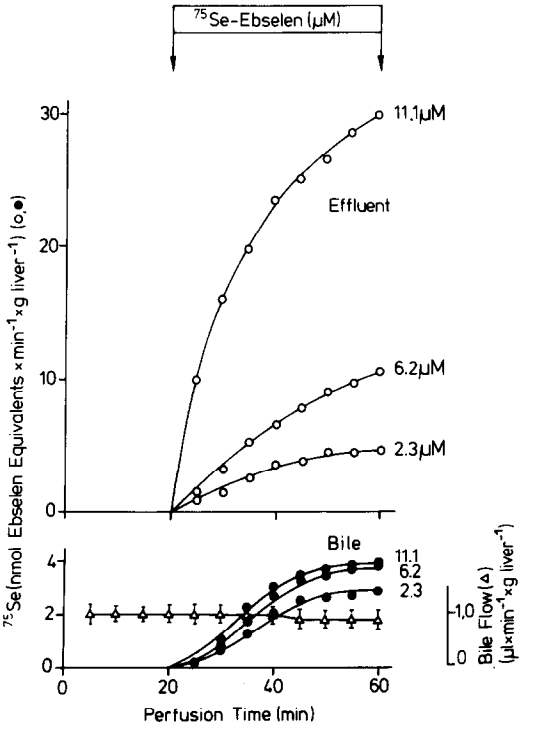


Fig. 1. Release of $^{75}$Se-compounds into effluent perfusate and bile upon the infusion of $^{75}$Se-ebselen in perfused liver. Values are means from 2–4 different experiments.

### *HPLC analysis of ebselen and its metabolites I–V from effluent and bile (see Fig. 2 for chemical structures)*

Isolated rat liver was perfused with 6.3 μM ebselen for 40 min. Bile and effluent were collected during this time period and prepared for analysis as described in Materials and Methods. The elution profiles obtained by HPLC are illustrated in Fig. 3.

A separation of synthetic reference compounds (50 μM) is shown in Fig. 3A.

The compounds were: E, ebselen (retention time, t = 12.8 min); I, 2-methylselenobenzanilide (t = 15.3 min); IV, 2-glucuronylselenobenzanilide (t = 5.4 min); V, 2,2'-Diselenobisbenzanilide (t = 20.5 min).

Table 1. Distribution of the amount of $^{75}$Se-ebselen equivalents in liver, bile and effluent

| | $^{75}$Se-ebselen equivalents (μmol per 40 min) | | |
|---|---|---|---|
| Infused amount | 3.2 (2) | 8.6 ± 0.3 (4) | 15.5 (2) |
| Liver | 1.3 (2) | 4.4 ± 0.5 (4) | 5.4 (2) |
| Bile | 0.7 (2) | 1.1 ± 0.2 (4) | 1.2 (2) |
| Effluent | 1.1 (2) | 2.9 ± 0.2 (4) | 8.3 (2) |

Values are means for two different perfusions or means ± SEM for four different experiments. Liver wet weight was 7.0 ± 0.3 g.

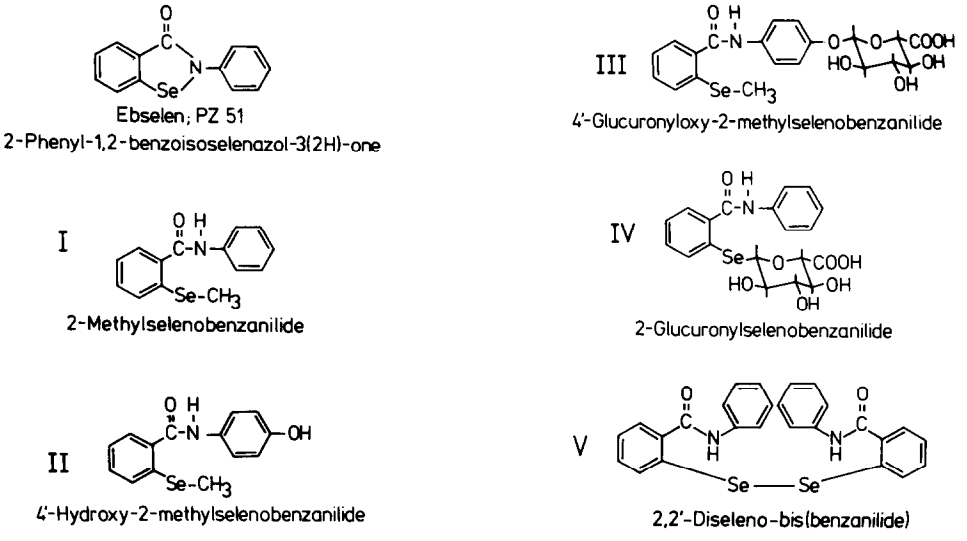


Fig. 2. Chemical structures of ebselen and of metabolites of ebselen I–V.

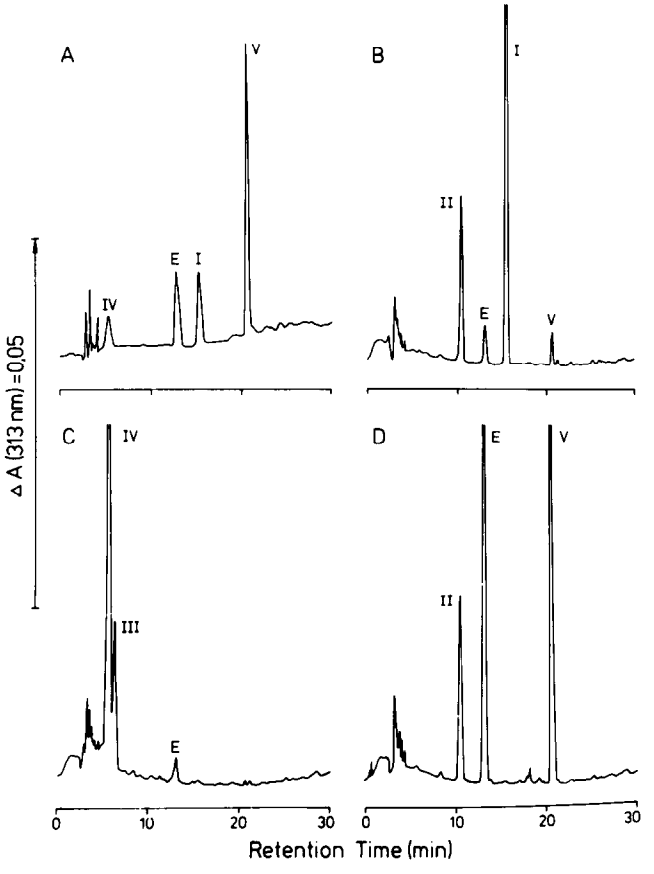


Fig. 3. HPLC elution profiles for ebselen and derivatives. (A) Synthetic reference compounds of organoselenium compounds at 50 μM concentrations; (B) metabolites of ebselen in the effluent perfusate; (C) metabolites of ebselen in the bile; and (D) metabolites of ebselen obtained after hydrolysis of the bile extract with beta-glucuronidase. E = ebselen and numbers I–V denote different metabolites of ebselen as shown in Fig. 2.

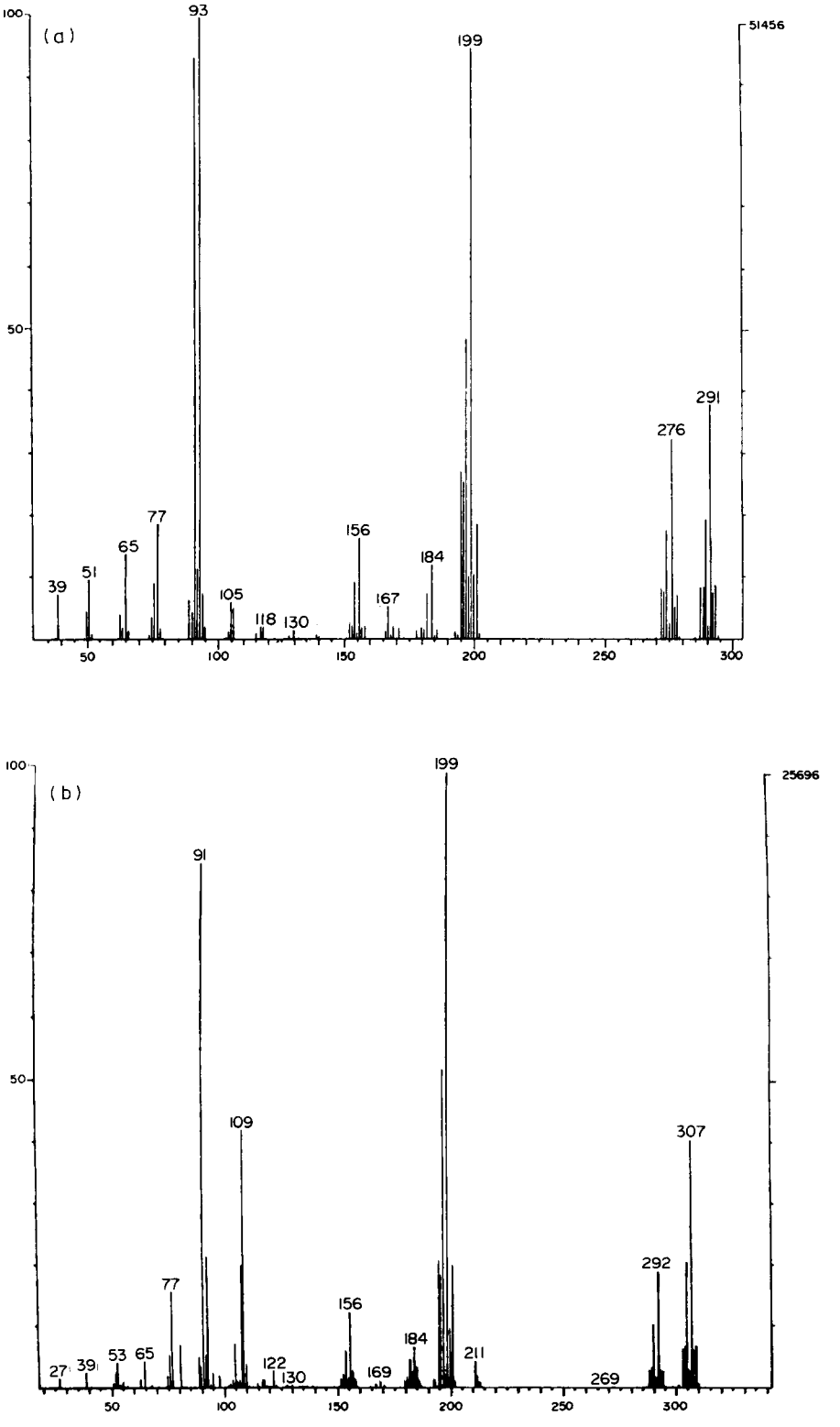


Fig. 4. (*continued on facing page*).

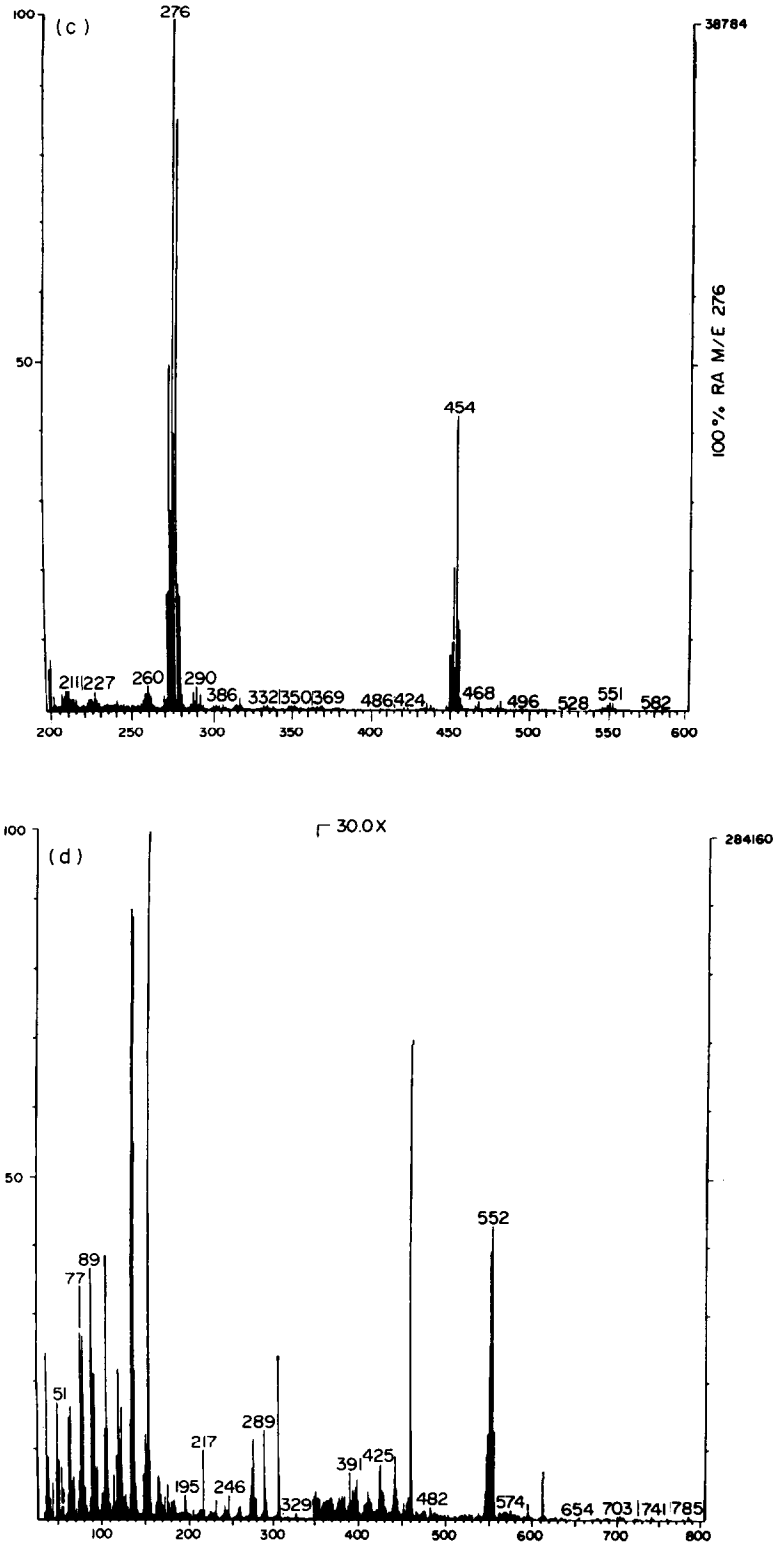


Fig. 4. Mass spectra of ebselen metabolites isolated from HPLC chromatography of samples from experiments shown in Fig. 3. (a) Metabolite I; (b) metabolite II; (c) metabolite IV, and (d) metabolite V. For further details, see Materials and Methods and text.

Figure 3B shows a chromatogram of the effluent extract. Four peaks were identified by coinjection of the respective synthetic reference compounds (Fig. 3A) and/or mass spectrometry (see below). These were ebselen and its metabolites I, II and V (II = *N*-(4-hydroxyphenyl)-2-methylselenobenzamide, t = 10.4 min). The respective amounts found in the pooled effluent, following infusion of 8.1 μmol ebselen for 40 min, were 0.07 μmol for ebselen, 1.55 μmol for metabolite I, 0.53 μmol for metabolite II and 0.08 μmol for metabolite V, expressed in ebselen equivalents. This adds up to 2.23 μmol ebselen equivalents in the pooled effluent perfusate (cf. Table 1).

Figure 3C shows the elution profile of a bile sample. Ebselen and the metabolites III and IV were found; the respective amounts were 0.10, 0.29 and 0.75 μmol, adding up to 1.14 μmol ebselen equivalents observed in the bile.

Figure 3D shows a chromatogram after treatment of a bile sample with beta-glucuronidase. Metabolites III and IV were no longer observed, indicating a total hydrolysis of conjugates. The sample contained ebselen and metabolite V, the diselenide of ebselen, generated by the hydrolysis, and the metabolite II, which was also observed in the effluent. The results obtained after hydrolysis with beta-glucuronidase imply that ebselen and metabolite V originated from the conjugate compound IV, and metabolite II originated from metabolite III, *N*-(4-glucuronyloxy)-2-methylselenobenzamide. Only two glucuronate conjugates appear to be present in the bile, whereas no sulfate conjugates were found in the bile. In agreement with this, no additional selenium compound was found after incubation with sulfatase (not shown). The amount of selenium compounds obtained after hydrolysis was similar to the amount before hydrolysis.

*Mass spectrometry of ebselen metabolites obtained from perfusion experiments*

*Metabolite I* (2-*Methylselenobenzanilide*). The molecular weight of I (Fig. 4A) is shown by the peak at $m/z = 291$, which is accompanied by the fragment signal at $m/z = 276$. The difference of 15 mass units, indicative of a methyl group, is due to a facile cleavage of the selenium methyl bond. This was encountered in all mass spectra of compounds containing this functional group. Alternatively, fragmentation of the amide bond took place, yielding an intensive peak at $m/z = 199$. All described ions obviously contained selenium, as can be deduced from the typical isotope pattern.

*Metabolite* II (N-(4-*hydroxyphenyl*)-2-*methylselenobenzamide*. The mass spectrum of metabolite II (Fig. 4B) is similar to that of metabolite I. The increase by 16 mass units in the molecular ion ($m/z = 307$) indicates the presence of an additional oxygen in the molecule. It can be located in the anilide moiety, as the typical fragment for the aniline group is shifted from $m/z = 93$ (aniline) to $m/z = 109$ (hydroxyaniline). The identity of metabolite II was confirmed by HPLC, where it coeluted with the synthetic reference compound.

*Metabolite* IV (2-*Glucuronylselenobenzanilide*). The very polar metabolite IV could not be evaporated without thermal decomposition. In the EI-mass spectrum only ions were observed which correspond to ebselen ($m/z = 275$). To circumvent these difficulties, we applied fast-atom-bombardment (FAB) ionisation. In a glycerol matrix a FAB-spectrum of IV was obtained (Fig. 4C) with sufficiently intensive ions in the higher mass range. The ion at $m/z = 454$ correspond to the molecular weight of a selenoglucuronide. The loss of the glucuronide moiety is reflected in the ion cluster at $m/z = 276$. IV is indistinguishable from a synthetic sample both by mass spectrometry and by HPLC.

*Metabolite* V (2,2'-*diselenobisbenzanilide*). Metabolite V also underwent thermal decomposition. Information about its molecular weight was obtained only with FAB-MS (Fig. 4D) using 3-nitrobenzylalcohol as FAB-matrix. A molecular ion cluster around $m/z = 552$ with the expected isotope pattern of selenium containing compounds was detected. The identity of V was shown by comparison with a synthetic sample.

## DISCUSSION

*Hepatic metabolism of ebselen*

The results demonstrate that ebselen is metabolized by rat liver cells. The metabolites found all share the common characteristic that the selenazole ring has been opened by the cleavage of the Se–N bond. Apparently, the putative intermediate product, e.g. a selenodisulfide with glutathione(*S*-(2-phenylcarbamoyl-phenylselenyl)-glutathione), is labile. The metabolism after ring-opening involves methylation of the selenium moiety to form metabolite (I), 2-methylselenobenzanilide, or glucuronidation to form metabolite (IV), 2-glucuronylselenobenzanilide. While the latter is released into the bile, the former (I) undergoes further metabolism or is excreted across the sinusoidal membrane into the effluent.

The metabolism of (I) includes hydroxylation at the phenyl ring in the *para* position to form metabolite (II) and the glucuronidation of metabolite (II) to form metabolite (III). The latter may again be excreted across the canalicular membrane into bile. This conclusion is supported by other experiments in which synthetic metabolite (I) was infused and hydroxylation and subsequent glucuronidation were observed (II and III), but no metabolite IV was detected (data not shown).

The formation of metabolite (V) in bile treated with beta-glucuronidase is thought to result from the dimerization of the unstable hydrolysis product, 2-selenoylbenzanilide.

*Chemical aspects: relation to selenium metabolism*

Inorganic selenium compounds as well as a number of organoselenium compounds such as selenocysteine share a final metabolic pathway, with dimethyl selenide and trimethylselenonium as known excretory products [16]. A pathway of biosynthesis of dimethyl selenide from hydrogen selenide has been worked out [17]. The methylation reactions are thought to be catalyzed by the microsomal thiol *S*-methyltransferase that methylates hydrogen sulfide [18, 19]. Ebselen may share this methylation pathway, and in fact the methylated compounds are the major metabolites.

The *p*-hydroxylation of metabolite I is most probably catalyzed by cytochrome P-450, but this has not yet been further investigated. The subsequent *O*-glucuronidation (metabolite III) is an expected step as catalyzed by UDP-glucuronyltransferases [20]. However, the Se-glucuronidation (metabolite IV) is a novel reaction. Apparently, Se-glucuronidation and Se-methylation are competing reactions, with 0.75 μmol metabolite IV formed versus a total of 2.37 μmol methylated compounds. Four general categories of glucuronides have so far been established, the *O*-, *N*-, *C*- and *S*-glucuronides [20, 21] and Se-glucuronides thus form a fifth category. The enzyme properties associated with the generation of Se-glucuronides are likely to resemble those observed for S-glucuronides, but relatively little information is as yet available on these [22, 23].

*Biological aspects: relation to selenium metabolism*

Previous work has shown that the Se in ebselen is not bioavailable. This conclusion stems from the observation that $^{75}Se$ from $^{75}Se$-ebselen, in contrast to $^{75}Se$-selenite, is not available for selenoprotein synthesis in mouse liver [2], and that ebselen given orally for 5 days does not raise GSH peroxidase activity in Se-deficient mouse macrophages [6]. This would also explain the high oral $LD_{50}$ of 6.8 g/kg (in mouse) and the very low toxicity of ebselen. Therefore, the organoselenium compound, ebselen, must be viewed differently from other compounds which readily enter the Se-pool.

*Acknowledgement*—Excellent technical assistance was provided by Maria Zimmer.

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