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TRANSPORT OF EBSELEN IN PLASMA AND ITS TRANSFER TO BINDING SITES IN THE HEPATOCYTE

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Abstract—*In vivo* transport in plasma and *in vitro* transfer of ebselen to binding sites in the hepatocyte were studied. More than 90% of intravenously administered ebselen in mouse plasma is bound by selenium–sulfur bonds to reactive thiols in serum albumin. In *in vitro* experiments the uptake of [^{14}C]-ebselen from a complex prepared with bovine serum albumin (BSA) was determined in isolated perfused rat liver. Radioactive ebselen metabolites were excreted into bile. In isolated hepatocytes, radioactivity was bound to all subcellular organelles. Ebselen is transferred from the BSA complex to membrane-associated proteins after reductive cleavage of the Se–S bond effected by endogenous protein thiols. In contrast, when proteins were separated by dialysis membranes, ebselen transfer from its BSA complex occurred only in the presence of externally added reductants. Among the physiological reductants tested, ebselen release from the BSA complex was highest with glutathione (75%) and lowest with ascorbic acid (less than 10%). Quantitative release of ebselen from its BSA complex was only achieved by the combined action of reductant, notably 2-mercaptoethanol, and guanidine thiocyanate, suggesting that ebselen interacts with proteins by covalent Se–S bonds as well as by ionic charge interactions.

Key words: ebselen; selenoorganic compound; albumin; hepatocytes; thiols; protein binding

Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2*H*)one, is a heterocyclic selenoorganic compound that mimics the catalytic activity of GSH† peroxidase (EC 1.11.1.9‡) *in vitro* [1–3] and exhibits anti-inflammatory activity *in vivo* [4].

Ebselen metabolism has been studied in isolated hepatocytes, in perfused rat liver and in whole organisms [3]. It is characteristic for all detected metabolites that the isoselenazole ring is reductively opened by cleavage of the Se–N bond [5, 6]. Metabolic studies have shown that in liver, ebselen is either reduced by thiols to 2-selenylbenzanilide [7] or reacts with endogenous thiol groups in proteins to form protein selenodisulfides [8]. After further transformation of 2-selenylbenzanilide, it is excreted as glucuronidated or methylated metabolites [9]. The effect of interference of ebselen with sulfhydryl groups in proteins is only partly understood. *In vitro* binding of ebselen to proteins was reported for bovine serum albumin [10], cytosolic protein [11] and membrane-bound protein [12]. It is assumed that rapid binding of ebselen to serum albumin results in a physiological transport form of ebselen as albumin complex in blood [10]. Intracellular effects of covalent binding of ebselen to essential thiol groups in proteins are the noncompetitive inhibition of glutathione *S*-transferases [11] and monooxygenases [7] and the inhibition of IP_3-induced calcium release [8].

Though the hepatic metabolism of ebselen and the mechanism of its catalytic action have been intensively studied, little is known about the vascular transport and uptake mechanism of ebselen by the liver or other organs. This study aims to contribute to understanding further the transport of ebselen in plasma, its subsequent transfer to binding sites in the hepatocyte and the nature of the ebselen protein bond.

MATERIALS AND METHODS

Materials. Male animals (NMRI mice/Wistar II rats) maintained under standard conditions were from the Animal Department, University of Düsseldorf. Ebselen and [^{14}C]ebselen (367 MBq × mmol^{-1}) uniformly labeled in the aniline moiety were kind gifts from Rhône-Poulenc-Nattermann (Cologne, F.R.G.). Dihydrolipoic acid was a gift from Asta Pharma AG (Frankfurt, F.R.G.). Sephadex G-10 and Sephacryl S-300 superfine were obtained from Pharmacia (Uppsala, Sweden), BSA, fraction V, and GSSG reductase from Boehringer (Mannheim, F.R.G.), NEM and DTE from Merck (Darmstadt, F.R.G.). Cellulose dialysis membranes (molecular cut-off of approx. 12.5 kDa) were from Serva (Heidelberg, F.R.G.).

In vivo administration of [^{14}C]*ebselen.* [^{14}C]Ebselen (1.2 μmol) dissolved in 20 μL *N*,*N*-dimethylformamide was injected under ether anaesthesia into the tail vein of a male NMRI mouse (40 μmol ebselen/kg body weight). The animal was killed by an excess of pentobarbital after 30 min. Blood was

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† Abbreviations: BSA, bovine serum albumin; DTE, dithioerythritol; GSH, glutathione; GSSG, glutathione disulfide; NEM, *N*-ethylmaleimide.

‡ Enzymes: glutathione peroxidase, EC 1.11.1.9; glutathione *S*-transferase, EC 2.5.1.18; GSSG reductase, EC 1.6.4.2; LDH, lactate dehydrogenase, EC 1.1.1.27.

collected from the portal vein and immediately centrifuged at 1000 *g* for 5 min to obtain the plasma fraction.

Separation of plasma protein fractions. Plasma proteins in the supernatant (0.8 mL) were separated by gel filtration on a column of Sephacryl S-300 superfine (2 × 90 cm) with water as eluent. Radioactivity and absorbance at 280 nm were determined in the collected 1 mL fractions. The albumin peak was identified by cellulose acetate electrophoresis with BSA as standard.

Preparation of a BSA-[^{14}C]ebselen complex. BSA (0.3 g) dissolved in 7.5 mL water and 6 μmol [^{14}C]ebselen (2.2 MBq) dissolved in 40 μL *N,N*-dimethylformamide (molar excess of ebselen about 1.4-fold) were incubated in a shaking water bath at 37° for 15 min. Free ebselen was removed from the mixture by gel filtration on a Sephadex G-10 column (2 × 80 cm) equilibrated with water. The eluted complex was identified by its radioactivity and absorbance at 280 nm, and was calculated to contain 0.3 mol ebselen/mol BSA. The radioactive BSA–ebselen complex was stable when stored frozen at −20°.

Perfusion of rat liver. Perfusion of isolated rat liver was performed at 37° in a non-recirculating system using hemoglobin-free, Krebs–Ringer bicarbonate buffer pH 7.4, equilibrated with O_2/CO_2 (19:1,v/v) essentially as described previously [9]. The BSA–ebselen complex was continuously infused with a micropump.

Hepatocytes and cell fractions. Hepatocytes were isolated from the livers of male rats (220–250 g body weight) by recirculating collagenase perfusion as described [13]. Fractions from hepatocytes were prepared according to the cited methods: nuclei [14], mitochondria [15], microsomes [12] and plasma membrane vesicles (mixed membranes) [16]. All incubations were performed at 37° in 10 mM potassium phosphate buffer, pH 7.5, unless stated otherwise. [^{14}C]Ebselen was dissolved in *N,N*-dimethylformamide. Protein was determined according to Bradford [17] using BSA as standard.

RESULTS

Thirty minutes after the injection of 1.2 μmol of [^{14}C]ebselen into the tail vein of a male mouse, ebselen concentration in plasma was 1.3 μM. Figure 1 shows the fractionation of plasma proteins by gel filtration on Sephacryl S-300. More than 90% of radioactive ebselen determined in plasma is found in the albumin fraction (1.8 mmol ebselen/mol albumin).

To determine the hepatic uptake of ebselen from its albumin complex *in vitro* by the whole organ, isolated hepatocytes and cell organelles, experiments were performed with a BSA-[^{14}C]ebselen complex (prepared as described in Materials and Methods). The amount of ebselen in the complex, also used for ebselen–protein interaction studies, was approx. 0.3 mol ebselen/mol albumin.

The hepatic uptake of ebselen from the albumin complex and the release of ^{14}C-labeled ebselen metabolites were determined in an isolated rat liver perfused with the BSA-[^{14}C]ebselen complex (Fig. 2). After 40 min pre-perfusion of the liver, the BSA–ebselen complex was added to the influent perfusate continuously for 20 min at a concentration of 0.21 μM ebselen corresponding to an infusion rate of about 840 pmol/min/g liver. A constant bile flow of approx. 1.5 mL/min/g liver was monitored during the entire time of perfusion. Twenty minutes after starting the infusion of the BSA-[^{14}C]ebselen complex, a maximal release of approx. 8 pmol/min/g liver of radioactive ebselen metabolites was determined in bile. Only a minor fraction of ebselen was taken up as indicated by portal-caval measurements.

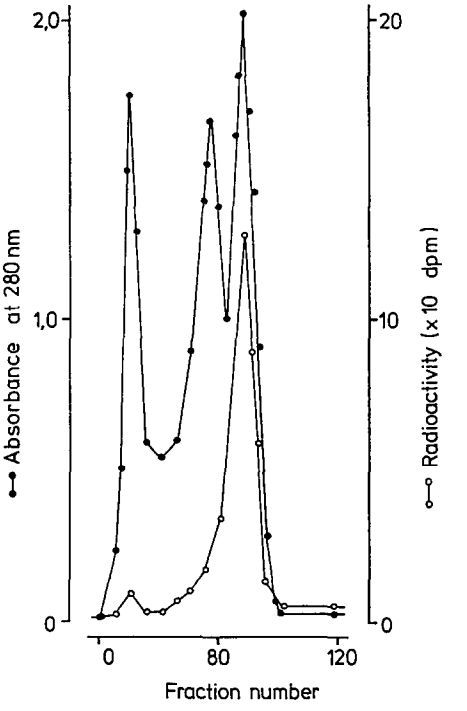

Fig. 1. Gel filtration of mouse plasma on Sephacryl S-300. Plasma was prepared from blood collected 30 min after i.v. administration of [^{14}C]ebselen.

The time-dependent uptake of free [^{14}C]ebselen (10 μM) by isolated hepatocytes at 37° is shown in Fig. 3. The distribution of ebselen was determined in sedimented hepatocytes and in the resulting supernatant after centrifugation. Maximal uptake of approx. 7 nmol ebselen per 10^6 hepatocytes was determined in sediments within the first minute of incubation at 37°, followed by a rapid decrease of intracellular radioactivity. The release of radioactive ebselen metabolites was determined in the supernatants. Viability of the hepatocytes was unaffected up to 40 min of incubation as demonstrated by constant low LDH activity in the supernatants.

The uptake of [^{14}C]ebselen from the BSA complex by isolated rat hepatocytes was studied and compared to the uptake of free ebselen. In Table 1 ebselen distribution is shown in cell fractions after incubation of hepatocytes with free or BSA-bound ebselen. The amount present after 5 min of incubation at 37° (ebselen concentration 10 μM) was 5 nmol/10^6 cells.

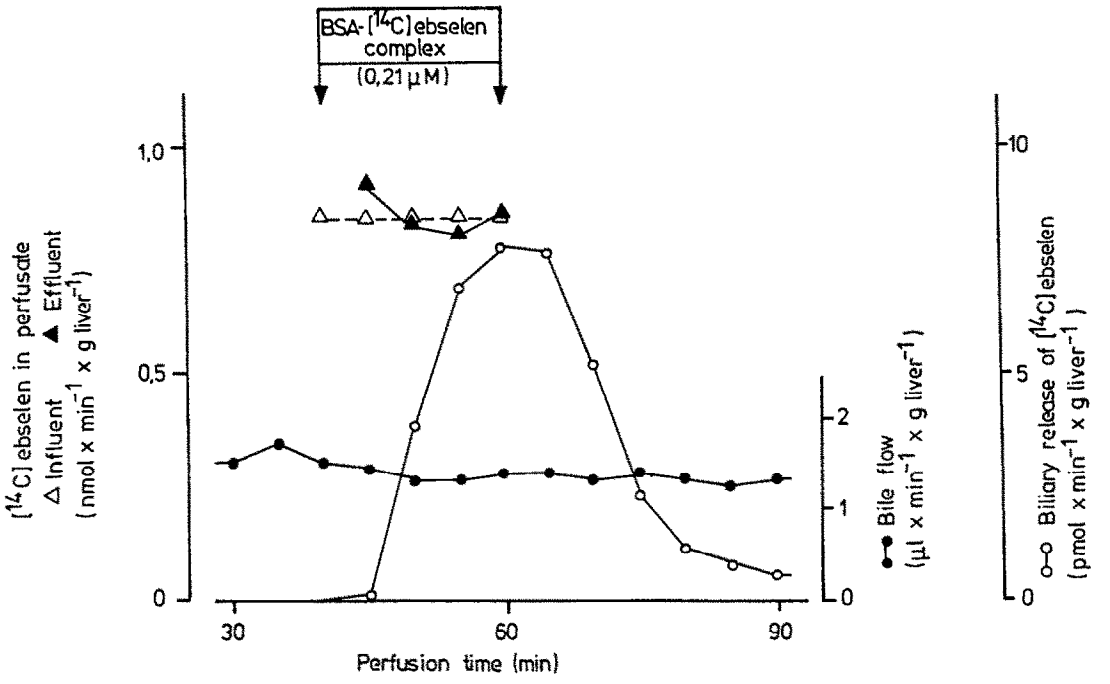

Fig. 2. Perfusion of an isolated rat liver with a BSA–[^{14}C]ebselen complex. Radioactivity in the influent and effluent perfusate samples and in bile as well as bile flow are presented.

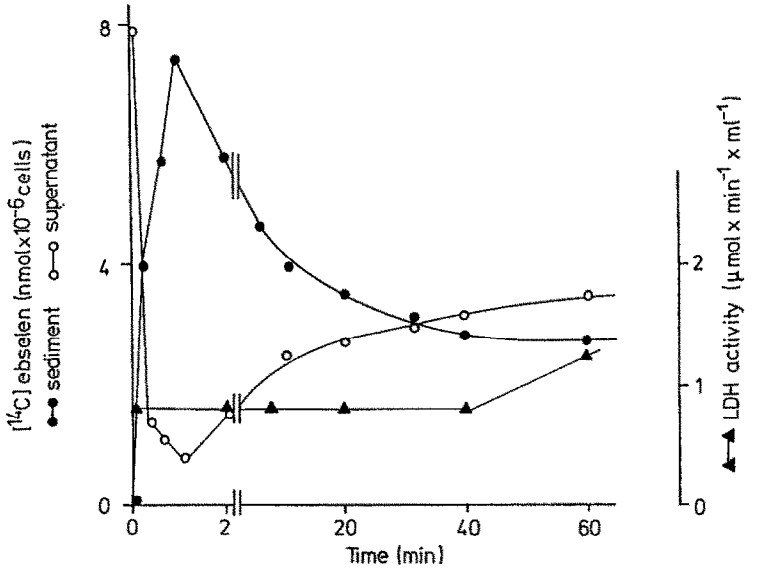

Fig. 3. Uptake of free [^{14}C]ebselen (10 μM) by isolated hepatocytes incubated in a bicarbonate-buffered salt solution equilibrated with O_2/CO_2 (19:1, v/v) at 37°. Radioactivity in supernatant and sedimented cells was determined in aliquots after centrifugation at 1000 *g* for 5 min.

No significant difference in the amount of ebselen uptake and cellular distribution was observed in isolated hepatocytes when ebselen was administered either in its free or BSA-bound form. The highest amount of ebselen (44%) was found in the cytosolic and the lowest (12%) in the microsomal fraction. The nuclear and mitochondrial fractions contained approx. 23%.

Ebselen uptake from the free and albumin-bound form was also studied in isolated nuclei and

Table 1. *In vitro* uptake of [^{14}C]ebselen by isolated rat hepatocytes from free or BSA-bound ebselen and its distribution in cell fractions

	Free ebselen	BSA-bound ebselen
	(nmol ebselen × 10^{-6} cells)	
Homogenate	5.2* (100%)	5.0 (100%)
Nuclear fraction	1.2 (23%)	1.1 (22%)
Mitochondrial fraction	1.3 (25%)	1.0 (20%)
Microsomal fraction	0.6 (11%)	0.6 (12%)
Cytosolic fraction	2.1 (41%)	2.3 (46%)

* Values are means of duplicate determinations.

Washed rat hepatocytes (1.2×10^6 cells/mL; viability 90%) were incubated with 10 μM ebselen in free or BSA-bound form for 5 min at 37° as described in Materials and Methods. After incubation cells were sedimented at 1000 *g* for 5 min, washed three times and disrupted by sonication (4 × 20 sec). Homogenates were fractionated by differential centrifugation.

Table 2. *In vitro* uptake of [^{14}C]ebselen by isolated rat liver nuclei and mitochondria from free or BSA-bound ebselen

	Uptake of [^{14}C]ebselen (pmol/mg protein)	
	Free ebselen	BSA-bound ebselen
Nuclei		
Suspension	1060 ± 75* (100%)	1010 ± 80 (100%)
Nuclei	950 ± 36 (90%)	574 ± 22 (57%)
Supernatant	109 ± 10 (10%)	520 ± 25 (51%)
Mitochondria		
Suspension	1021 ± 119 (100%)	961 ± 170 (100%)
Mitochondria	861 ± 30 (84%)	440 ± 11 (46%)
Supernatant	92 ± 6 (9%)	491 ± 30 (51%)

* Values are means ± SEM (N = 4).

Isolated rat liver nuclei (11 mg protein/mL) and mitochondria (27 mg protein/mL) were incubated with 10 μM ebselen in free or BSA-bound form for 5 min at 37°. After centrifugation and repeated washing, radioactivity and protein concentration were determined in sediments and supernatants.

mitochondria. As shown in Table 2, the amount of ebselen present in nuclei and mitochondria showed no significant difference when calculated per mg protein. However, uptake was dependent on the ebselen form administered, being approx. 900 pmol/mg protein for free ebselen and approx. 500 pmol/mg for the BSA complex.

To investigate a possible special function of the plasma membrane in ebselen uptake from the BSA complex by cells and organelles, the uptake by rat liver plasma membrane vesicles was determined by incubation of membranes for 5 min at 37° with the BSA complex ranging from 0 to 10 μM ebselen (Fig. 4). Indeed, ebselen uptake from the BSA complex (10 μM ebselen) by plasma membranes was three times higher (1.4 nmol/mg protein) than by isolated nuclei or mitochondria (0.5 nmol/mg protein). When membranes were preincubated for 5 min with 1 mM *N*-ethylmaleimide to block SH-binding sites, no ebselen transfer was observed (data not shown).

In the following experiments the mechanism of ebselen transfer from the BSA complex to cytosolic rat liver proteins was determined under experimental conditions in order to prevent direct interaction between complex and cytosol. Equal volumes of cytosol and BSA–ebselen complex (10 μM ebselen) were incubated at 37° separated by a dialysis membrane with a molecular cut-off of approx. 12.5 kDa. Approximately 190 pmol of radioactive ebselen from the BSA–[^{14}C]ebselen complex were bound per mg cytosolic protein after 60 min at 37° and only 10 pmol were bound when the cytosol was intensively dialysed before the transfer experiment. As shown in Fig. 5, ebselen transfer depends on time and on the presence of GSH. A linear dependence on GSH concentration was determined over a range of 0–100 μM GSH (data not shown).

From this experiment it was concluded that ebselen is transferred from protein to protein after preceding ebselen release by reductive cleavage of the selenium–sulfur bond. A further experiment was conducted to investigate the effect of different reductants. The release of ebselen by reductants from the BSA complex (10 μM ebselen) is shown in Fig. 6. At 5 mM concentrations of the reductants, a high release of approx. 70% ebselen was measured for dithioerythritol, sodium borohydride and the physiological reductant GSH, and a low release of about 10% for ascorbate.

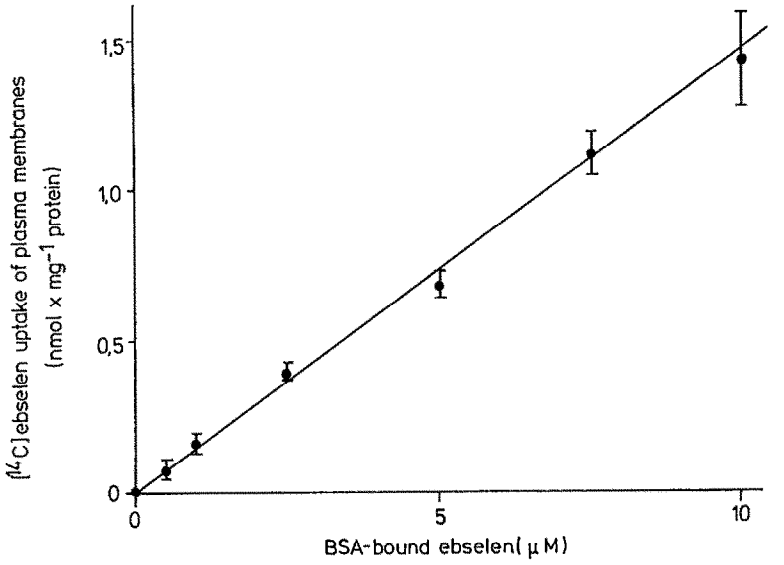

Fig. 4. Binding of [^{14}C]ebselen from its BSA complex by rat liver plasma membrane vesicles. Plasma membrane vesicles (mixed membranes, 1.2 mg protein/mL) were incubated with the BSA–[^{14}C]ebselen complex (1.8 mg protein/mL, 10 μM ebselen) for 5 min at 37°. Radioactivity and protein content in membranes were determined after repeated washings by vesiculation and centrifugation at 105,000 *g* for 45 min. Values are means ± SEM from four determinations.

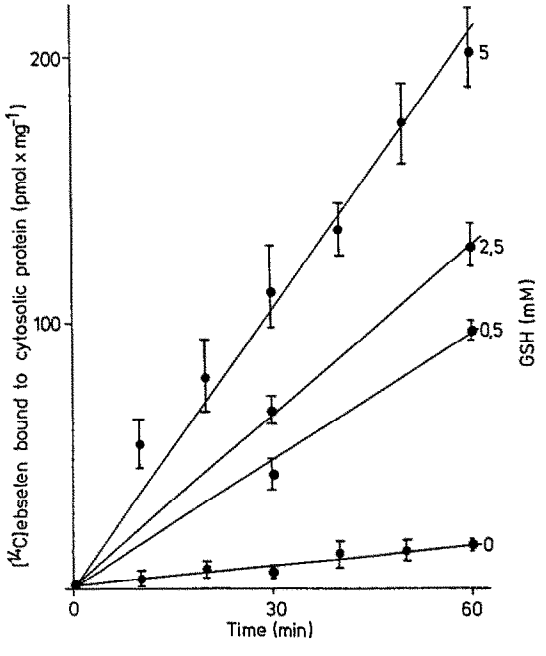

Fig. 5. Transfer of [^{14}C]ebselen from the BSA-bound complex (10 μM ebselen, 2.5 mg protein/mL) to dialysed rat liver cytosol (24 mg protein/mL) under the influence of GSH. Equal volumes of cytosol and the BSA–ebselen complex separated by a dialysis membrane were incubated at 37° after the addition of GSH. Cytosolic proteins were precipitated with 10% trichloroacetic acid, collected on glass fiber filters and washed with ethanol. Radioactivity of the filters was measured. Each value is the mean ± SEM from four determinations.

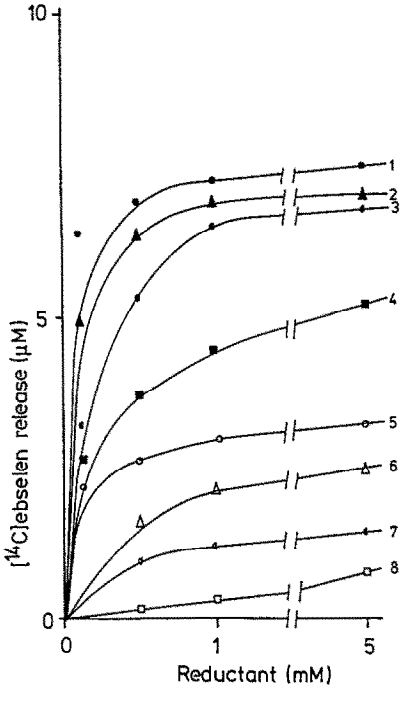

Fig. 6. Release of ebselen from a [^{14}C]ebselen–BSA complex (10 μM ebselen, 2.5 mg protein/mL) by different reductants. Incubation was for 10 min at pH 7.5 and 37°. Aliquots (200 μL) were extracted with 1 mL ethylacetate and centrifuged for 2 min at 10,000 *g*. Radioactivity was determined in the organic phase. The reductants are: 1 = dithioerythritol, 2 = GSH, 3 = sodium borohydride, 4 = dihydrolipoic acid, 5 = GSSG/GSSG reductase/NADPH, 6 = sodium dithionite, 7 = sodium thiosulfate, 8 = ascorbic acid.

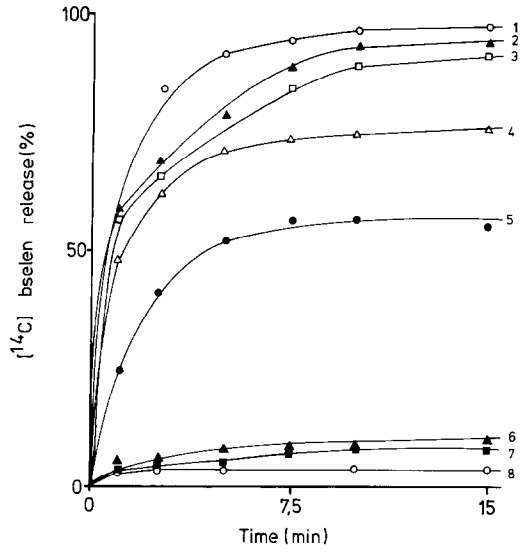

Fig. 7. Release of [^{14}C]ebselen from a BSA–[^{14}C]ebselen complex (10 μM). Conditions as in Fig. 6. 1 = 5 M guanidine thiocyanate/ 5 mM 2-mercaptoethanol, 2 = 5 M guanidine thiocyanate/ 1 mM 2-mercaptoethanol, 3 = 5 M guanidine thiocyanate, 4 = 5 mM 2-mercaptoethanol, 5 = 1 mM 2-mercaptoethanol, 6 = 1 mM *N*-ethylmaleimide, 7 = ethylacetate, 8 = phosphate buffer, pH 7.5.

As no quantitative release of ebselen from the BSA complex was achieved by reductants, conditions affecting salt bonds were examined. In Fig. 7 the time-dependent effect of 2-mercaptoethanol is shown in combination with 5 M guanidine thiocyanate. Similar to other thiols as dithioerythritol or GSH, approx. 75% of ebselen was released by 5 mM 2-mercaptoethanol after 10 min at 37°, but nearly 100% by 5 mM 2-mercaptoethanol in combination with 5 M guanidine thiocyanate.

Direct evidence for the electrostatic interaction of ebselen with albumin was provided by examining its interaction with thiol blocked albumin. After pretreatment with *N*-ethylmaleimide the albumin SH-groups were quantitatively saturated as determined with Ellman reagent. When *N*-ethylmaleimide pretreated albumin was incubated with a 3.5-fold molar excess of [^{14}C]ebselen, 0.02 mol ebselen was bound per mol *N*-ethylmaleimide albumin as compared to 0.33 mol ebselen bound per mol native albumin.

DISCUSSION

It is currently held that ebselen is transported in the organism bound to proteins. In blood, albumin is the general transport protein that contributes significantly to the distribution and metabolism of hydrophobic ligands [18]. In model studies using bovine serum albumin Nomura *et al.* [10] observed a rapid binding of ebselen to albumin. After reductive opening of the isoselenazole ring [5, 6], ebselen reacts rapidly with the sulfhydryl group of cysteine 34, the only reactive thiol group in serum albumin [19] to form a selenosulfide [20]. The *in vitro* result [10] was confirmed *in vivo*, for more than 90% of [^{14}C]ebselen administered by i.v. injection to a mouse was observed to be bound to the albumin fraction after 30 min (Fig. 1). A determined plasma concentration of 1.25 μM ebselen is low when compared to the calculated capacity of albumin to bind 600 μM ebselen by selenium–sulfur bonds [12]. The low plasma concentration of labeled ebselen bound to albumin *in vivo* may result from rapid equilibration between plasma albumin and the tissues.

In vitro uptake of ebselen from an albumin–ebselen complex by rat hepatocytes was studied using a BSA–[^{14}C]ebselen complex. The animal species supplying the albumin in the ebselen complex seems to be irrelevant, since the albumin amino acid sequences are highly conserved in human, bovine and rat serum albumin, especially concerning cysteine residues [21]. When an isolated rat liver was perfused with the BSA-bound ebselen, hepatic metabolism of BSA complex derived ebselen was demonstrated by excretion of radioactive ebselen metabolites into bile (Fig. 2). The main ebselen metabolite excreted into bile during liver perfusion is a Se-glucuronide [9]. Maximal release of the ebselen metabolite into bile was approx. 1% of the complex-bound ebselen in the influent perfusate when calculated for the steady state (Fig. 2).

Ebselen uptake by isolated rat hepatocytes and its distribution in cell fractions was similar when ebselen was administered free or as BSA complex. The highest ebselen content was in the cytosol. In the presence of low-molecular-weight thiols such as GSH there seem to be equilibria allowing ebselen to be transferred to the cytosolic compartment where glutathione *S*-transferases are suggested to be the preferred ebselen-binding proteins [11]. In contrast, when isolated organelles such as nuclei or mitochondria were incubated with free or bound ebselen, the uptake from the BSA complex was only approx. 50% as compared to free ebselen. When plasma membranes, the compartment with direct contact with the albumin–ebselen complex in blood, were incubated with the radioactive albumin complex *in vitro*, a transfer of radioactive ebselen from albumin to membrane proteins was observed, dependent on ebselen concentration (Fig. 4). This protein–protein transfer occurred without the addition of reductants. However, it was not observed when membranes were preincubated with the thiol reagent NEM. From these findings it is concluded that ebselen is released from the albumin complex by reductive cleavage of the selenium–sulfur bound by the free sulfhydryl groups of membrane-associated proteins. Ebselen uptake per mg protein from a BSA complex (10 μM ebselen) by plasma membranes is approx. 3-fold higher than uptake by isolated organelles.

The molecular nature of the ebselen moiety transferred between proteins has not yet been identified. Ebselen reacts with endogenous thiol groups in proteins to form selenyl sulfides which might constitute a storage form of ebselen and eventually be responsible for the transport of the drug [6]. After reductive release of ebselen from the protein complex by low-molecular-weight thiols and presumably by protein thiol groups, ebselen can react rapidly with the thiol according to a proposed

scheme [22] to yield a selenyl sulfide which, in turn, can react with excess thiol to an ebselen selenol intermediate [23].

No significant transfer of ebselen from the albumin complex to cytosolic proteins was observed, when direct interaction between BSA complex and cytosolic proteins was prevented by a dialysis membrane. However, in the presence of GSH as reductant, a time-and dose-dependent release of ebselen from the BSA complex and a transfer of free ebselen to cytosolic proteins was seen (Fig. 5).

Though direct transfer of ebselen by protein–protein interaction in the absence of reductants was observed, their presence was indeed required for indirect protein transfer. To elucidate these findings, the effect of low-molecular-weight reductants on the release of ebselen from its BSA complex was determined. The most powerful reductants tested were dithioerythritol, sodium borohydride and GSH. Though ebselen release from the BSA complex (10 μM) was determined at concentrations of 5 mM reductants, maximal ebselen release was only about 75%. This is in line with the observation described for the non-competitive inhibition of glutathione *S*-transferase activity by irreversible ebselen binding to essential enzyme sulfhydryl groups, which was only partly restored by 5 mM thiols [11]. Other physiological reductants such as ascorbic acid, dihydrolipoic acid [22] or the GSH regenerating system of GSSG reductase/NADPH [23] exhibit only a minor effect on ebselen release.

To examine the incomplete release of ebselen from its BSA complex, 2-mercaptoethanol as strong reductant was used in combination with 5 M guanidine thiocyanate. The effect of 5 mM 2-mercaptoethanol—approx. 75% ebselen release—was similar to the effect of 5 mM GSH, but ebselen release from the BSA complex was 100% when the complex was incubated with 5 mM 2-mercaptoethanol/ 5 M guanidine thiocyanate.

From these results one may conclude that ebselen is bound to proteins not only by covalent selenium–sulfur bonds but also by ionic bonds. The essential step for the formation of both types of binding is the reductive opening of the isoselenazole ring by cleavage of the Se–N bond. Besides the formation of a covalent selenium–sulfur bond between ebselen and protein, a peptide-like bond is formed in the ebselen molecule by ring opening which generates ionic charges capable of interacting with charged groups in protein.

The stabilization of the protein–ebselen complex by covalent and electrostatic interactions may be advantageous for certain functions of the complex. In plasma with a GSH concentration of 10 μM [24] the selenosulfide-bond of the BSA–ebselen complex is not affected, but in the hepatocyte at 5 mM GSH concentration electrostatic interactions may become of relevance for protein–ebselen interactions.

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