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FURTHER CHARACTERIZATION OF MEMBRANE PROTEINS INVOLVED IN THE TRANSPORT OF ORGANIC ANIONS IN HEPATOCYTES

COMPARISON OF TWO DIFFERENT AFFINITY LABELS: 4,4′-DIISOTHIOCYANO-1,2-DIPHENYLETHANE-2,2′-DISULFONIC ACID AND BROMINATED TAURODEHYDROCHOLIC ACID

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4,4′-Diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid (H_2DIDS) known as an irreversible inhibitor of the anion transport in red blood cells (Cabantchik, Z.I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311–330) blocks also the uptake of bile acids and of some foreign substrates in isolated hepatocytes (Petzinger, E. and Frimmer, M. (1980) Arch. Toxicol. 44, 127–135). [^{3}H]H_2DIDS was used for labeling of membrane proteins probably involved in anion transport of rat liver cells. The membrane proteins modified in vitro by [^{3}H]H_2DIDS were compared with those labeled by brominated taurodehydrocholic acid. The latter is one of a series of suitable taurocholate derivatives, all able to bind to defined membrane proteins of hepatocytes and also known to block the uptake of bile acids as well as of phallotoxins and of cholecystographic agents (Ziegler, K., Frimmer, M., Möller, W. and Fasold, H. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 319, 254–261). The radiolabeled proteins were compared after SDS-electrophoresis with and without reducing agent present, solubilization by detergents, two-dimensional electrophoresis and after separation of integral and peripheral proteins. Our results suggest that the anion transport system of liver cells cannot distinguish between bile acids and the anionic stilbene derivative (DIDS). The labeling pattern for both kinds of affinity labels was very similar. Various combinations of separation techniques gave evidence that the radiolabeled membrane proteins are not subunits of a single native channel protein.

Introduction

4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and H_2DIDS well known as potent inhibitors of the anionic transport in red blood cells [1,2], decrease the uptake of bile acids by isolated liver cells [3]; in addition DIDS blocks the inward transport of several foreign substrates (e.g., Iodipamide) and of phallotoxins [4]. With respect

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; Temed, *N,N,N′,N′*-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; [^{35}S]BTC, brominated [^{35}S]taurodehydrocholic acid; [^{3}H]H_2DIDS, 4,4′-diisothiocyano-1,2-diphenyl[^{3}H]ethane-2,2′-disulfonic acid.

to the specific binding of [^{3}H]H_2DIDS to the band 3 proteins in red cell membranes it is of interest, which proteins of the liver plasma membrane are labeled by this reagent. DIDS and H_2DIDS were found to inhibit irreversibly the phalloidin response of isolated hepatocytes in a concentration and time dependent manner [4]. Binding of H_2DIDS to plasma membranes was correlated to the degree of inhibition of the phalloidin response [4]. DIDS inhibited the uptake of cholate and of demethylphalloin to a similar degree [3]. At that time, the specific radioactivities of [^{3}H]H_2DIDS available were too low for the identification of the labeled proteins in membranes after incubation of isolated cells. In the present study we used a [^{3}H]H_2DIDS preparation with a 100-fold higher specific radioactivity (1 Ci/mmol). Thus we were able to compare labeling of liver plasma membranes with [^{3}H]H_2DIDS and with brominated [^{35}S]taurodehydrocholic acid ([^{35}S]-BTC).

In a previous series of experiments we studied several affinity labels derived from bile acids or from cholecystographic agents [5]. In particular brominated taurodehydrocholate inhibited the uptake of [^{14}C]cholic acid in a concentration-dependent manner. The same label binds to five membrane proteins after incubation either of liver cells or of isolated plasma membranes [6]. Pretreatment of liver cells with DIDS prevented subsequent binding of brominated taurodehydrocholate. Similar results were obtained by several photoaffinity labels derived from taurocholate, too [7].

So far tested all naturally occurring bile acids and also their covalently binding derivatives inhibit the inward transport of phallotoxins in liver cells and the in vitro response of isolated hepatocytes to phalloidin [8,9]. Cholate was found to be a competitive inhibitor of the phalloidin uptake [10]. We suspected therefore that the bile acid channel is needed for the uptake of phalloidin [11]. Another proof for this hypothesis is the fact that AS 30D ascites hepatoma cells, which are defective in bile acid uptake [12] are also unable to transport and to response to phalloidin in vitro [13]. Membranes of these cells bind only negligible amounts of brominated taurodehydrocholate. These findings gave evidence for the specificity of affinity labeling of membrane proteins in normal hepatocytes.

Because of similar effects of (photo)affinity labels derived from bile acids and H_2DIDS we suspected that both kinds of labels might modify greatly identical proteins in liver membranes. This paper argues that the anion transport system of liver cells cannot distinguish between bile acids and the anionic stilbene derivative DIDS.

Materials

The synthesis and characterization of [^{35}S]BTC and of [^{3}H]H_2DIDS were described earlier [6,14]. SDS and urea were purchased from Merck, Darmstadt, F.R.G. PMSF, Triton X-100, bovine serum albumin, Temed, acrylamide, *N,N'*-methylenebis(acrylamide) and Coomassie brilliant blue G were from Serva, Heidelberg, F.R.G.; Sephadex IEF from Pharmacia, Sweden; Aquacides from Calbiochem; ovalbumin from Miles and trypsin from Boehringer, Mannheim, F.R.G. All other reagents used were of the highest purity grade available commercially.

Methods

Membrane preparation. Plasma membranes were prepared from rat liver according to Touster et al. [15], with the following modifications: After homogenization of the liver tissue 1 mM PMSF was added. The discontinuous sucrose gradient centrifugation was performed in a Vertical rotor (Beckman, VTi 50) for 2 h.

Affinity labeling of isolated plasma membranes. Isolated plasma membranes in phosphate buffered saline (2 mg/ml) were incubated for 15 min at room temperature with 0.3 μM [^{3}H]H_2DIDS (1 Ci/mmol) or for 30 min at 37°C with 20 μM [^{35}S]BTC (140 μCi/mmol). Unbound label was removed by excessive washing.

Membrane solubilization. Labeled plasma membranes were resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100 and 1 mM PMSF at a concentration of 2 mg of protein per ml. They were incubated at 4°C for 60 min and then centrifuged at 100000 $\times g$ for 120 min. Under these conditions 70–80% of the membrane proteins and 80–90% of radioactivity were solubilized.

Protein determination. Protein was determined

according to Lowry et al. [16] using bovine serum albumin as a standard. For determinations in the presence of detergent (Triton X-100) the protein was precipitated with 10% trichloroacetic acid at 95°C for 5 min, collected by centrifugation and washed once with dioxan. The alkaline copper tartrate solution for the procedure of Lowry contained 0.5% SDS.

Extraction of extrinsic or peripheral membrane proteins. [^{35}S]BTC- and [^{3}H]H_2DIDS-labeled plasma membranes were resuspended (10 mg protein/ml) in 1 mM EDTA pH 8.0, and incubated for 1 h at 4°C. Extractable proteins were separated from intrinsic proteins by centrifugation for 1 h at 100 000 × *g*.

SDS-gel electrophoresis under reducing and non-reducing conditions. [^{35}S]BTC- and [^{3}H]H_2DIDS-labeled plasma membranes and Triton X-100 solubilized samples were dissolved in SDS buffer with or without varying concentrations of mercaptoethanol and heated for 3 min at 95°C before SDS gel electrophoresis and fluorographic analysis of labeled polypeptides. Two-dimensional gel analysis of labeled proteins without reduction in the first and with reduction in the second dimension were performed according to Hynes et al. [17].

Separation of labeled, Triton X-100 solubilized plasma membrane proteins by isoelectric focusing linked to SDS-polyacrylamide gel electrophoresis. Analytical isoelectric focusing was carried out according to Bhakdi et al. [18] with the following modifications: The gel rods for IEF were cast without urea to keep the solubilized proteins in a native state. The ampholines used were in the pH range of 3.5–10 and 4–6, respectively.

The pH gradient was measured with a WTM DIGI 610 pH-meter, by cutting the gel rods into 4 mm slices and placing them in 2 ml aqua bidest for 3 h at room temperature.

The gel rods for the second dimension in SDS were incubated for 2 h at 37°C in 5 ml of 2% SDS, 5% mercaptoethanol in Tris-HCl buffer (pH 6.7) with 0.004% Bromophenol blue. The equilibrated gels were subjected to electrophoresis in the second dimension in 10% acrylamide slabs (10 × 16 × 0.15 cm, acrylamide/bisacrylamide 74 : 1, v/v) in the presence of 0.1% SDS. Fluorographic visualization of the labeled polypeptides was performed according to Laskey et al. [19]. The isoelectric focusing gels and the second dimension SDS-polyacrylamide gels after electrophoresis were soaked in 50% methanol for 12 h to remove the ampholines. The gels were stained with Coomassie brilliant blue (G-250, Serva) and scanned with a Gilford Instruments Spectrophotometer, equipped with a linear transport accessory.

Sucrose-density-gradient centrifugation. Step gradients (3.9 ml) were prepared from 7.5% to 35% sucrose in 25 mM Tris-HCl (pH 7.4)/0.02% NaN_3, and 0.05% Triton X-100. Applied Triton X-100 solubilized proteins were centrifuged at 4°C in a Beckman VTi 50 vertical rotor at 42 000 rpm for 2 h. Twenty fractions were collected.

Preparative flat-bed electrofucusing. Preparative isoelectric focusing in layers of granulated gels of Sephadex IEF was carried out in the LKB 2117 Multiphor as originally described [20]. The [^{35}S]BTC- and [^{3}H]H_2DIDS-labeled Triton X-100 solubilized proteins were included in the original gel slurry. Electrofocusing was run for 16–21 h with a constant power of 8 W (initial conditions 200 V, 40 mA, final conditions 800 V, 10 mA). The separated zones were eluted with 5 ml of 10 mM Tris-HCl, pH 7.4/4 M urea/0.5 mM PMSF/0.5% Triton X-100, and dialyzed for two days against 10 l of 10 mM Tris-HCl/0.5 mM PMSF/0.02% NaN_3 with two changes.

Polyacrylamide gel electrophoresis in the presence of 0.1% Triton X-100 linked to SDS-polyacrylamide gel electrophoresis. Protein samples were subjected to electrophoresis in 5% polyacrylamide slab gels (10 × 16 × 0.15 cm) containing 0.1% Triton X-100 (separation at pH 9.5) according to Maurer [21] and Dulaney et al. [22]. The second dimension SDS-polyacrylamide gel electrophoresis was done as described above.

Antisera production and fused rocket immunoelectrophoresis. Rabbit antisera to rat liver plasma membrane proteins were raised according to Bjerrum [23]. Fused rocket immunoelectrophoresis was done as described by Axelsen [24].

Results

Characterization of membrane proteins covalently labeled with [^{3}H]H_2DIDS or [^{35}S]BTC

Isolated membrane vesicles (2 mg/ml in phos-

phate-buffered saline pH 7.4) were incubated with 0.3 μM [^{3}H]H_2DIDS (15 min 20°C) or 20 μM [^{35}S]BTC (30 min 37°C). There was no difference of the electrophoretic pattern of Coomassie blue stainable proteins from liver plasma membranes which had been labeled with one of both compounds (data not shown). That means that the applied concentrations of both labels were unable to produce splitting or cross-linking of membrane proteins to a marked degree. Fig. 1 shows the modified proteins (molecular weights for [^{3}H]H_2DIDS labeled proteins 110000, 67000, 60000, 54000, 50000, 46000, 43000, 38000, 32000, 24000).

After treatment of membranes with [^{3}H]H_2DIDS all modified proteins except the 43000, 46000 and the 24000 polypeptides were labeled to a well reproducible extent. In some experiments the 46000 protein was not resolved from the broad band of 50000 and 54000 proteins or not detectable.

The latter phenomenon holds, too, for the 43000 and 24000 proteins. Whether this fact results from some proteolysis remains to be established. (All experiments were carried out in the presence of 1.0 mM PMSF). The 50000 and 54000 proteins often represent a broad band in sections of SDS slab gels. Because of the band width its accurate molecular weight was difficult to determine. The broad band may be composed of more than one component (50000 + 54000). For quantification of the binding of [^{3}H]H_2DIDS, SDS slab gels were sliced and the radioactivity was determined. The 54000 protein (50000–54000 band) represents the major [^{3}H]H_2DIDS-labeled component (Fig. 2).

As expected from earlier studies with [^{35}S]BTC, membranes prepared from AS-30D ascites hepatoma cells did not bind significant concentrations of [^{3}H]H_2DIDS either. However, after incubation with a 2-fold higher concentration of [^{3}H]H_2DIDS a 43000 protein was markedly labeled and a polypeptide of 54000 was labeled

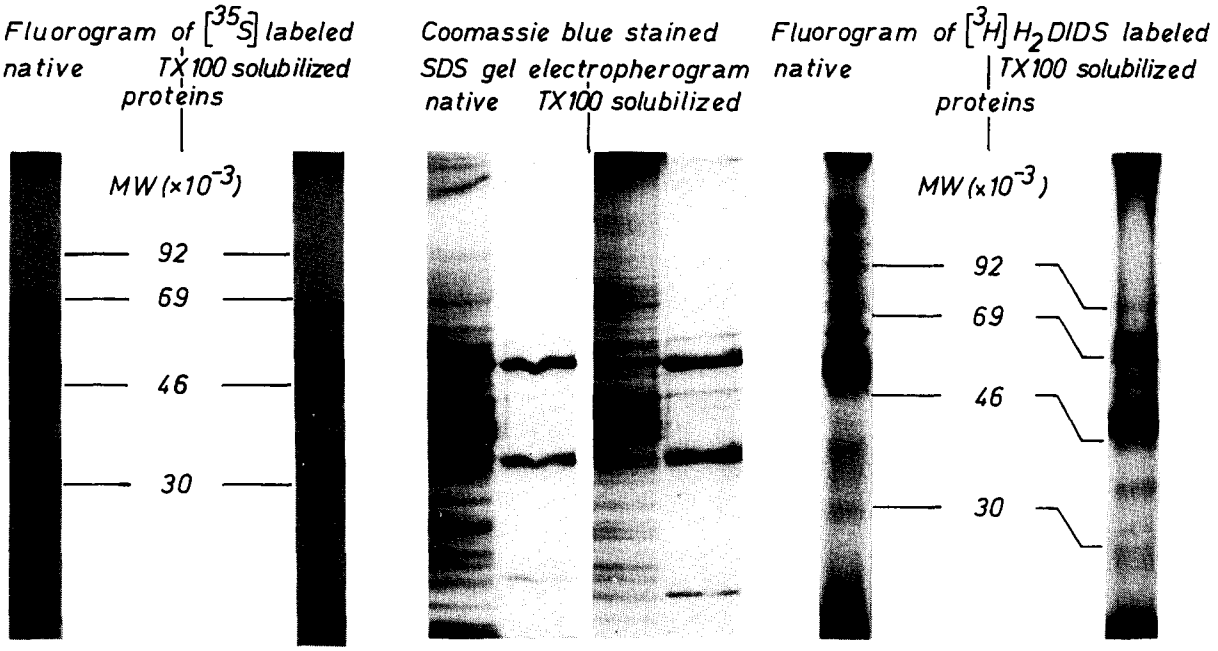


Fig. 1. Electrophoretogram and fluorogram of proteins labeled with brominated [^{35}S]taurodehydrocholic acid and [^{3}H]H_2DIDS. Isolated liver plasma membranes from rat liver were treated either with [^{35}S]BTC or with [^{3}H]H_2DIDS. After removal of the unbound labels SDS-polyacrylamide gel electrophoresis was performed. The distribution of radioactivity was determined by fluorography. In other experiments the labeled proteins were first solubilized in 1% Triton X-100 (1 h, 4°C). After high speed centrifugation the supernatant was used for SDS gel electrophoresis. The labeled proteins were visualized by fluorography. TX 100, Triton X-100.

only to a slight and not well reproducible extent (Fig. 2). Preincubation of membranes with H_2DIDS prevented the incorporation of [^{3}H]H_2DIDS in a concentration-dependent manner (Fig. 2). The specificity of labeling with [^{3}H]H_2DIDS was ascertained by the fact that the radioactivity associated with the polypeptides was significantly reduced when the membranes were preincubated with a natural substrate (taurocholic acid) with competitive inhibitors (Iopodate [25]) or

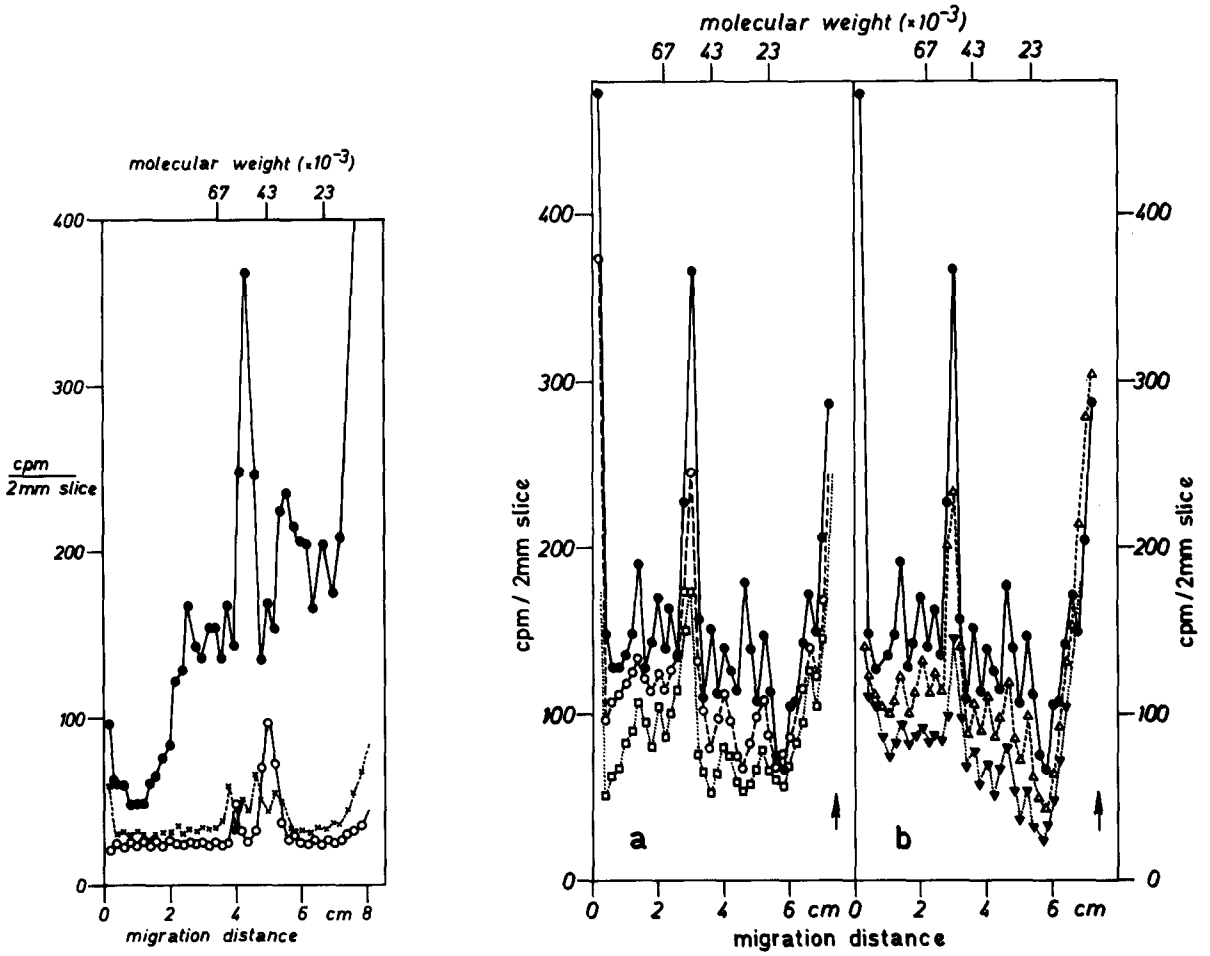


Fig. 2. Distribution of [^{3}H]H_2DIDS in plasma membranes prepared from normal rat livers and from AS-30D hepatoma cells after SDS electrophoresis. Isolated plasma membranes from rat liver were labeled with 0.3 μM [^{3}H]H_2DIDS with (×– – –×) and without (●———●) preincubation with 200 μM unlabeled H_2DIDS. Aliquots were washed and subjected to SDS gel electrophoresis. AS-30D ascited cell plasma membranes were incubated with a 2-fold higher concentration of [^{3}H]H_2DIDS. The labeled proteins were processed as described above (○———○). The radioactivity profile was detected after sectioning SDS slab gels. Molecular weight standards are: bovine serum albumin 67000, ovalbumin 43000 and trypsin 23000.

Fig. 3. Protection of [^{3}H]H_2DIDS labeling by taurocholic acid, Iopodate and isothiocyanatobenzamidocholic acid. Isolated plasma membranes in 200 mM phosphate buffer pH 7.4 (2 mg/ml) were preincubated 10 min at 20°C without (●———●) or with 0.5 mM, 0.8 mM taurocholic acid, 0.5 mM Iopodat and 0.5 mM isothiocyanatobenzamidocholic acid. After addition of 0.3 μM [^{3}H]H_2DIDS the membranes were incubated for 15 min. The labeling was terminated by addition of 100 mM Tris-HCl. The labeled plasma membranes were washed to remove unbound [^{3}H]H_2DIDS. 100 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis. The distribution of radioactivity was determined by sectioning the SDS slab gels and counting the radioactivity. (a) Inhibition by taurocholic acid: ○———○, 0.5 mM; □———□, 0.8 mM. (b) Inhibition by Iopodate (△– – –△) or isothiocyanatobenzamidocholic acid (▼– – –▼). The arrows indicate the buffer front.

with irreversible inhibitors of the bile acid uptake (isothiocyanatobenzamido cholic acid) (Fig. 3). Attention must be called to the pH of the incubation medium. Binding of [^{3}H]H_2DIDS increases with increasing pH. This could be due to conformational changes of membrane proteins or to the deprotonation of amino groups by alkalinization. We used therefore 200 mM potassium/sodium phosphate buffer (pH 7.4) for all protection studies. The labeling pattern obtained with the above buffer was not different from the pattern in phosphate-buffered saline. (Compare Figs. 2 and 3).

Protection of [^{3}H]H_2DIDS labeling by phalloidin under similar conditions produced controversial data and needs further careful investigation. In plasma membrane preparations of liver cells phalloidin binds to microfilaments inside the membrane [26]. We don't know whether the vesicles are altered by high phalloidin concentrations needed in protection experiments.

The profile of ^{35}S-labeled membrane proteins (molecular weights 67000, 60000, 54000, 50000, 46000, 38000, 32000, 24000) was very similar to that of [^{3}H]H_2DIDS with the exception of the 110000 and 43000 proteins.

The fluorographic analysis of [^{35}S]BTC labeled proteins identifies three major bands (50000, 54000, and 67000). A 46000 protein was not always well resolved from the 50000–54000 proteins. The latter proteins could hardly be separated by sectioning SDS slab gels or gel rods. In an earlier publication we reported the occurrence of a 49000 protein. Its mole weight was determined by sectioning SDS gel rods.

Fluorography of SDS slab gels shows that the former 49000 band is composed of two proteins with mole weight of 50000 and 54000, respectively. Furthermore, [^{35}S]BTC binds to a 60000, 38000, 32000 and a 24000 protein less intensely. The 24000 protein couldn't be detected in all experiments. Both labels bound to the same polypeptides, although with characteristic differences in the extent of labeling.

Separation of integral and associated membrane proteins after labeling of liver plasma membranes

Channel components are thought to be integral membrane proteins, whereas more hydrophilic (glyco) proteins on the surface may be responsible for recognition and trapping of transported compounds. Therefore, labeling can principally occur on both types of proteins. In order to evaluate the localization of the labeled proteins we extracted plasma membranes with 1 mM EDTA at pH 8.0 (see Methods).

The [^{3}H]H_2DIDS-labeled 67000, 43000, 38000 and 32000 proteins as well as the [^{35}S]BTC-labeled 67000 and 38000 proteins remained in the supernatant after extraction and centrifugation at 100000 × g for 1 h (Fig. 4). These proteins may be extrinsic, whereas the labeled 60000, 54000, 50000 and 46000 polypeptides could only be solubilized in detergents, indicating that they are intrinsic components of the plasma membrane.

These results agree with previous studies by Öbrink et al. [27]. Proteins with the above mole

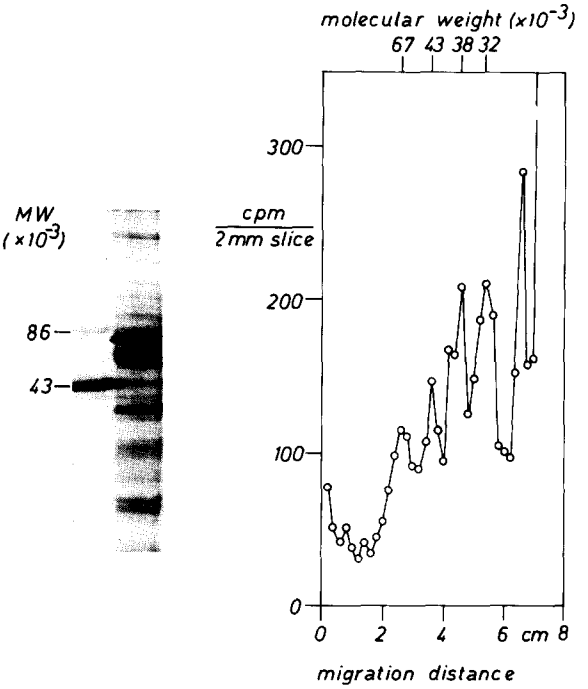


Fig. 4. EDTA-extractable [^{3}H]H_2DIDS- and [^{35}S]BTC-labeled extrinsic proteins of isolated plasma membranes. [^{3}H]H_2DIDS- or [^{35}S]BTC-labeled proteins were extracted with 1 mM EDTA pH 8.0 during 1 h at 4°C. After high speed centrifugation (1 h, 100000 × g) the labeled proteins in the supernatant were analyzed by SDS gel electrophoresis. The distribution of radioactivity in the gel was determined by sectioning SDS slab gels. Shown is a Coomassie blue stained SDS gel of the EDTA-extractable proteins and standard proteins and the radioactivity profile of [^{3}H]H_2DIDS-labeled proteins.

weights could be extracted by EGTA from isolated membrane vesicles. None of these proteins appeared in a EGTA perfusate from rat liver. Thus, it seems likely that these peripheral proteins are localized on the cytoplasmic side of the membrane.

Investigations on disulfide bonds between the labeled membrane proteins

Both [^{3}H]H_2DIDS and [^{35}S]BTC labeled proteins might be components of high molecular units linked by disulfide bridges. Therefore, fluorograms were compared after electrophoresis of membrane proteins with or without preceding mercaptoethanol treatment. No obvious differences in distribution of the radioactive bands were detected (Fig. 5). The pattern of labeled proteins was very similar after pretreatment with [^{3}H]H_2DIDS as well as with [^{35}S]BTC. Apparently, disulfide bridges do not exist. Further examination by two-dimensional electrophoresis gave the same results. In the first dimension the proteins were separated without mercaptoethanol, in the second dimension they were separated after reduction.

As shown in Fig. 6 all proteins separated by the techniques mentioned above are distributed along a diagonal; that means no splitting has occurred under reducing conditions. We conclude that no interchain S-S bonds may be present in the [^{3}H]H_2DIDS-labeled membrane proteins.

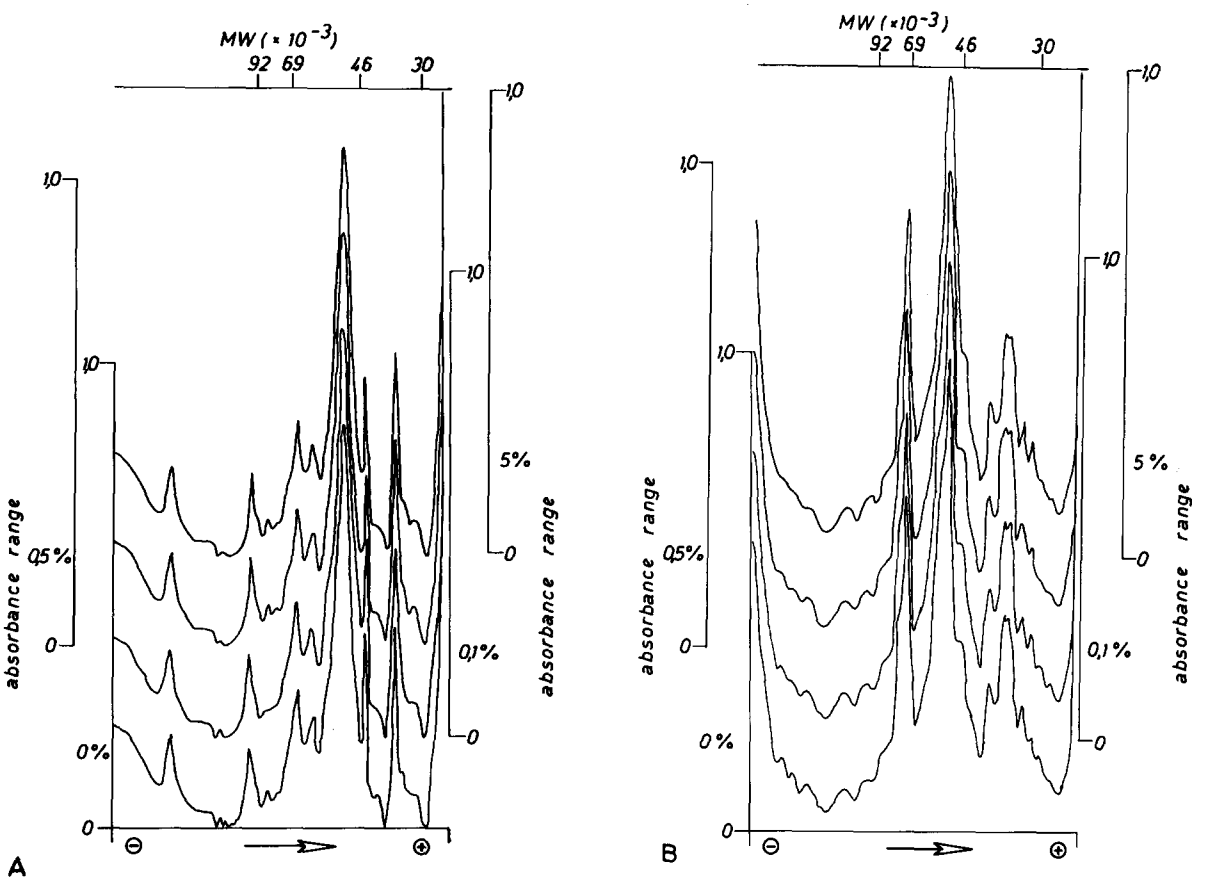
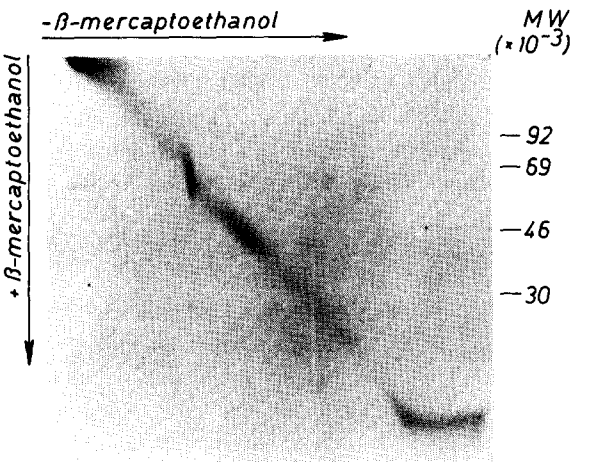


Fig. 5. SDS gel electrophoresis of [^{3}H]H_2DIDS- or [^{35}S]BTC-labeled liver plasma membrane proteins with and without reduction by β-mercaptoethanol. Isolated plasma membranes were labeled either with [^{3}H]H_2DIDS or with [^{35}S]BTC. The unbound label was removed by a standard washing procedure. The labeled proteins were analyzed either before (a) or after reduction with 0.1%, 0.5% or 5% β-mercaptoethanol by SDS gel electrophoresis and subsequent fluorography of the slab gels and densitometric analysis at 610 nm. (A) Densitogram of [^{3}H]H_2DIDS-labeled proteins. (B) Densitogram of [^{35}S]BTC-labeled proteins.



Solubilization of the [^{3}H]H_2DIDS- and the [^{35}S]BTC-labeled proteins and resolution by different two-dimensional electrophoretic techniques

For further characterization of the labeled proteins the plasma membranes were solubilized with three different detergents (Triton X-100 Nonidet

Fig. 6. Two-dimensional gel electrophoresis of [^{3}H]H_2DIDS-labeled plasma membrane proteins. Plasma membranes were labeled with [^{3}H]H_2DIDS. Aliquots were run on cylindrical gels without reduction (10% acrylamide). These gels were placed at the top of a slab gel and electrophoresed after reduction in the second dimension. Control experiments with or without reduction in both dimensions were performed (data not shown).

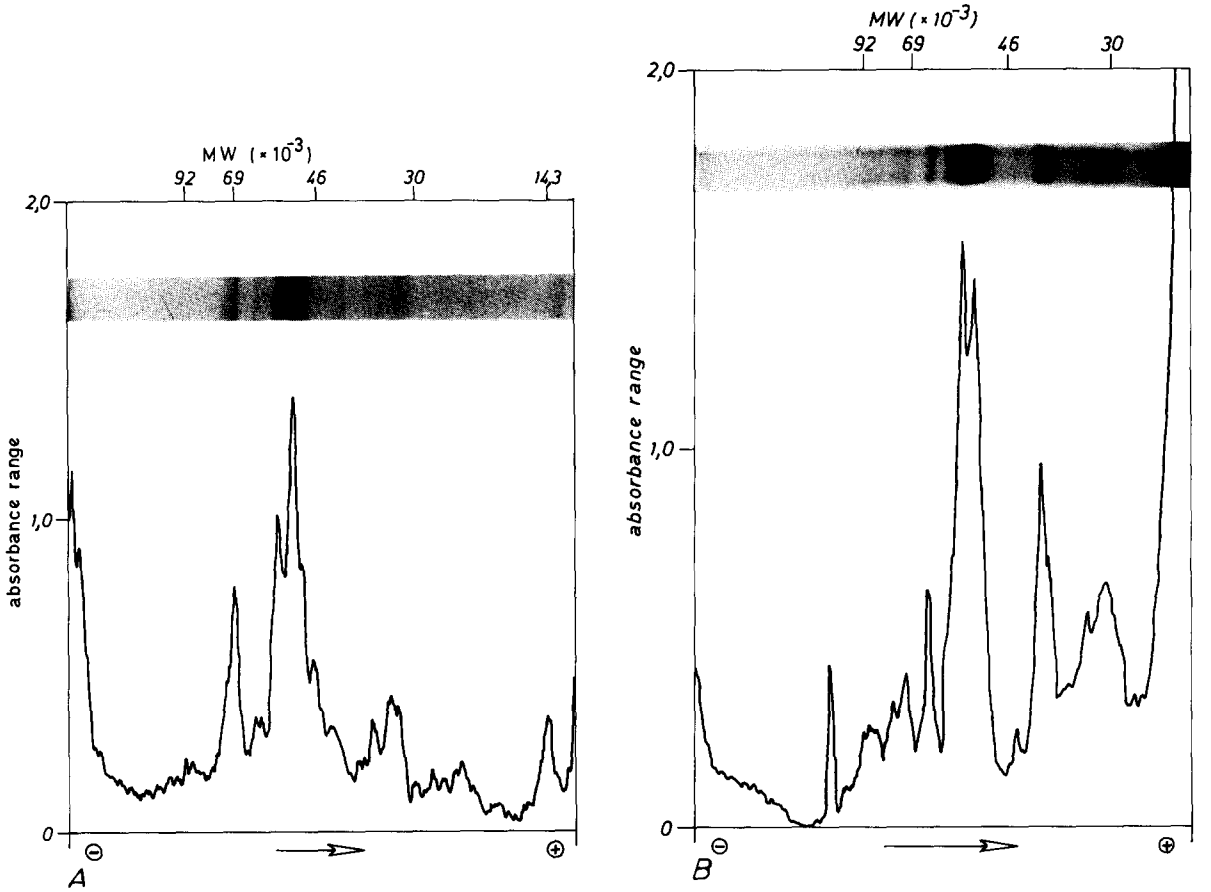


Fig. 7. Electrophoresis of Triton X-100 solubilized proteins of plasma membranes from rat liver. Comparison of membrane proteins labeled either with [^{35}S]BTC or with [^{3}H]H_2DIDS. [^{35}S]BTC- or [^{3}H]H_2DIDS-labeled plasma membranes were solubilized in 1% Triton X-100. The labeled proteins were visualized by fluorography and the radioactivity bound was quantitated by densitometric analysis of the fluorograms, at 610 nm. (A) Densitometric analysis of a fluorogram of [^{35}S]BTC-labeled proteins. (B) Densitometric analysis of a fluorogram of [^{3}H]H_2DIDS-labeled proteins.

P-40, taurodeoxycholic acid). The efficiency of solubilization was similar and the pattern of solubilized proteins was independent of the type of detergent used. For the following study we used Triton X-100 (detergent/protein ratio 5 : 1). The solubilized fraction was separated by high speed centrifugation. Fig. 7A shows a densitometric analysis of a fluorogram from [^{35}S]BTC-labeled Triton X-100 solubilized proteins after electrophoresis in SDS. Fig. 7B shows the same for [^{3}H]H_2DIDS-labeled proteins. The radioactivity profiles of [^{3}H]H_2DIDS and [^{35}S]BTC-labeled proteins before and after Triton X-100 solubilization are very similar (compare Figs. 1, 2 and 7). The [^{3}H]H_2DIDS-labeled 43 000 protein was found in the non-solubilized pellet after centrifugation. Small amounts of the 50000 and 54000 [^{35}S]BTC- and [^{3}H]H_2DIDS-labeled proteins also remained in the insoluble material (data not shown).

In order to find out whether the plasma membrane proteins retain their ability to bind [^{35}S]BTC after solubilization with mild detergents, plasma membranes were solubilized prior to affinity labeling. Fluorographic visualization of modified proteins gave the same results as above (data not shown). Therefore, treatment of membrane proteins with Triton X-100 did not disturb bile acid binding sites.

In earlier experiments [^{35}S]BTC- and DIDS-labeled proteins have been studied by one-dimensional electrophoresis only. In this system the labeled proteins were very similar after pretreatment with both types of labels. Additional studies with other techniques were needed to confirm the above parallelism. We used a combination of isoelectric focusing in the first dimension with subsequent SDS-electrophoresis in the second dimension. In the above system the labeled membrane proteins focused between pH 4 and 7. After sectioning of the first dimension isoelectric focusing gel rods radioactive peaks could be detected at pH values of 7.0, 6.3, 6.0, 5.5, 5.1, 4.5, 4.0 for [^{35}S]BTC-labeled proteins and at pH 7.1, 6.3, 6.0, 5.5, 5.1, 4.5 and 4.0 for [^{3}H]H_2DIDS-labeled ones. The radioactivity at pH 4.0 represents unbound

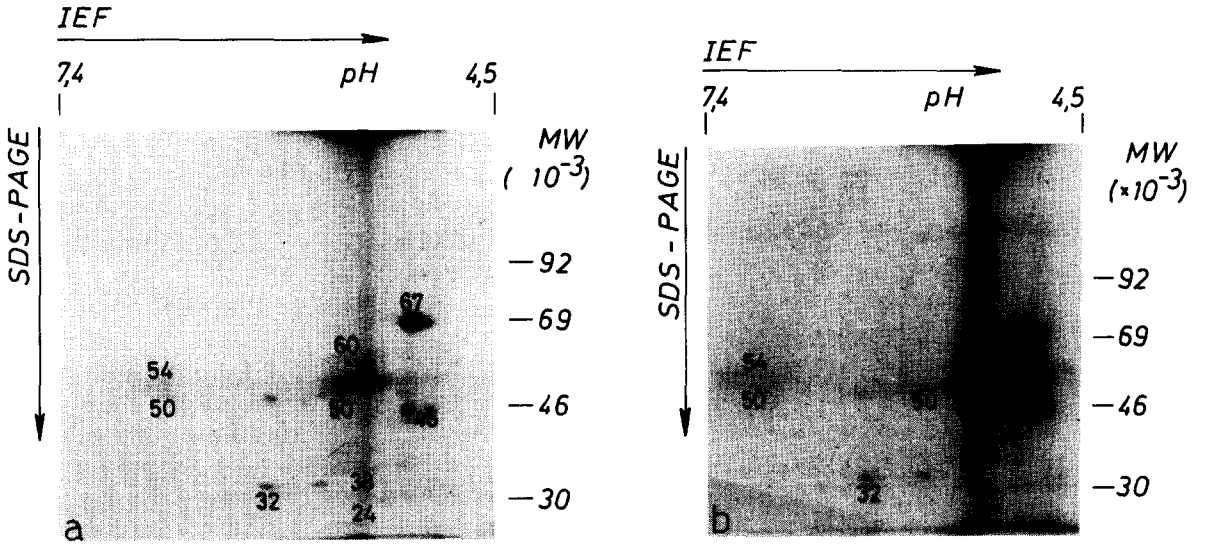


Fig. 8. Two-dimensional electrophoretic separation of [^{3}H]H_2DIDS- and of [^{35}S]BTC-labeled proteins. Comparison of fluorograms of two-dimensional slab gels. In all gels, the basic region is on the left, the acidic on the right. The pH ranges on the top of the figures were measured in the isoelectric focusing gels with the aid of a pH-electrode after sectioning the gel rods. Molecular weight standards used were [^{14}C]methylated 92500 phosphorylase B, 69000 bovine serum albumin, 46000 ovalbumin, 30000 carbonic anhydrase. Pattern obtained with 200 μg of [^{35}S]BTC-labeled proteins and 400 μg of [^{3}H]H_2DIDS-labeled proteins. The fluorogram with the [^{3}H]H_2DIDS-labeled proteins was exposed for 12 weeks, the ^{35}S-labeled ones for 4 weeks. The same pattern of spots could be obtained when urea was included in the first dimension isoelectric focusing. (a) [^{35}S]BTC-labeled proteins. (b) [^{3}H]H_2DIDS-labeled proteins.

label (holds for [^{3}H]H_2DIDS as well as for [^{35}S]BTC). Again the [^{35}S]BTC or [^{3}H]H_2DIDS-labeled proteins behaved very similar in the system used (pH range 3.5–10). The fluorographic analysis of the two-dimensional electrophoresis of [^{3}H]H_2DIDS- or [^{35}S]BTC-labeled proteins is shown in Fig. 8. The radioactive spots correspond to spots also seen in the Coomassie blue staining pattern. The position of these spots was reproducible in all experiments. Comparison of the localization of the ^{35}S-labeled (Fig. 8a) and the ^{3}H-labeled (Fig. 8b) spots demonstrate similarities of the binding proportions of both labels. Most of both labels (^{3}H or ^{35}S) appears to be fixed in a single spot representing a molecular weight of 67000. The heavily labeled and broad 50000–54000 SDS-subunits (seen in one-dimensional gels) focus in multiple spots. However, it is not fully understood whether aggregation of proteins in Triton X-100 or charge multiplicity of the 50000 and 54000 band is responsible for the described distribution. The inclusion of urea in the isoelectric

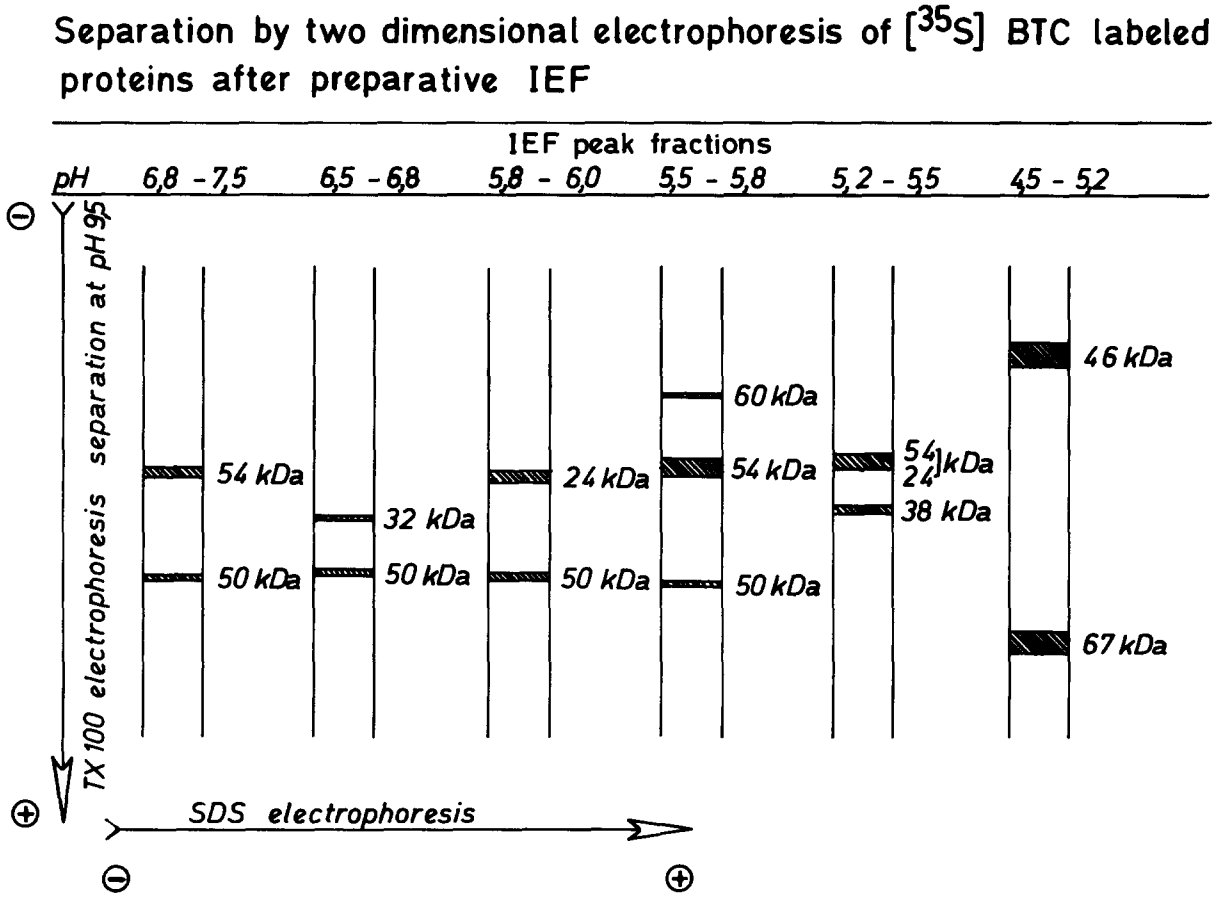


Fig. 9. [^{35}S]BTC-labeled plasma membrane proteins were solubilized with Triton X-100. After removal of the excess of Triton X-100 by sucrose gradient centrifugation, preparative isoelectric focusing in a granulated gel was performed. Radioactive peak fractions were detected between pH 7.0–4.0. The success of the separation was monitored by fused rocket immunoelectrophoresis. Radioactive peak fractions with confluent immunoprecipitates were pooled and separated by electrophoresis in Triton X-100 containing gels at pH 9.5 (5% acrylamide). These gels were then equilibrated in SDS buffer and placed on the top of a 0.1% SDS containing 10% acrylamide gel and run in the second dimension. The distribution of radioactive spots was analysed by fluorography. The scheme shows the results obtained with [^{35}S]BTC-labeled proteins. In the case of [^{3}H]H_2DIDS the only difference was the occurrence of an additional 110000 protein in the pH range of 5.8–6.

focusing gel did not modify the result. On the other hand a discrimination of the 50000 and 54000 bands was possible by isoelectric focusing. The 46000 spot cofocuses with the 67000 spot. The 32000 spot does so with the 50000, the 54000 with the 24000 spot and the 54000 and 50000 with the 60000 after labeling of membranes with [^{35}S]BTC. We had difficulties to localize the 110000 ^{3}H-labeled protein in the two-dimensional electrophoresis depicted, but in preparative isoelectric focusing fractions we detected the 110000 protein in the pH range of 5.8–6.

In order to decide whether the cofocusing labeled SDS subunits (e.g. the 67000 and the 46000) are subunits of the same native protein or whether they originate from different proteins with the same migration in isoelectric focusing we carried out the isoelectric focusing in a preparative scale. Thereafter the cofocusing proteins were separated according to molecular weight. This was achieved by a two-dimensional electrophoresis technique. The cofocusing native proteins were electrophoresed in Triton X-100 containing gels (pH 9.5) in the first dimension under non-denaturating conditions. The separation took place according to mole weight and to charge of the Triton X-100-solubilized native proteins at pH 9.5. Thereafter the first-dimensional Triton X-100 containing gels were equilibrated with SDS. The gels were placed on top of a SDS slab gel and electrophoresed in the second dimension under denaturating conditions. The labeled SDS-subunits were localized by fluorography of the SDS slab gel in the second dimension. We used this technique because of the multiplicity of peak fractions and the limited amounts of material. The cofocusing proteins, e.g. the 46000 and 67000 proteins, show different mobilities in the first-dimensional Triton X-100 electrophoresis. With this approach the cofocusing 46000 and 67000 proteins and the other cofocusing proteins (except in the case of the 54000 and 24000 proteins) could be separated (Fig. 9 [^{35}S]BTC).

These results were obtained for [^{3}H]H_2DIDS- as well as for [^{35}S]BTC-labeled proteins. They are further proof for the conclusion that both labels modify the same SDS subunits (mole weights) and that these SDS subunits originate from different native proteins.

Discussion

Our working hypothesis of a multispecific transport system for a series of organic anions and for phallotoxins originates from various transport studies on isolated liver cells [3,28,8,29]. Additional experiments showed that this multispecific system does not exist in intestinal membranes [30]. The intestinal system is highly specific for bile acids.

It is of great interest that affinity labels derived from bile acids modify proteins in intestinal membranes quite different from those shown in studies with liver cell plasma membranes [31]. The multispecificity of the bile acid channel in the liver cell membrane might be a suitable mechanism for extraction of foreign substrates from the portal blood. In contrast the high specificity for bile acids in intestinal cells prevents the uptake of unwanted foreign organic anions and of toxic cyclopeptides. It is therefore of interest to study the properties of both types of channels and their molecular compositions in particular. In this context one has to regard the fact that nearly all known translocating systems are oligomer.

The data presented in this study for the hepatocellular system give evidence for two facts: [^{35}S]BTC and [^{3}H]H_2DIDS, chemically different anionic compounds, bind to the same membrane proteins in liver plasma membranes (except for the 110000 and the 43000 protein). The differences are rather of quantitative than of qualitative nature. This might be due to the different mode of chemical reaction during covalent binding. [^{3}H]H_2DIDS binds preferentially to the 54000, [^{35}S]BTC to the 50000, 54000 and 67000 proteins. We feel that a molecular concept of a multispecific channel will develop from further studies with still other chemically different labels. Such strategies produce more information on the molecular level than kinetical uptake studies alone.

The second result of our study is that the proteins modified by both labels (DIDS and BTC) cannot be subunits of a single channel protein only. The 67000, 43000, 38000 and 32000 proteins, all susceptible to both affinity labels are probably peripheral membrane proteins. The question is whether they are associated with the integral units outside or inside the membrane. The

67000 protein was identified as albumin by immunoprecipitation [7]. It is probably not directly involved in the transport of organic anions. The 43000 protein was shown to comigrate with purified G-actin prepared from hog liver. This protein is insoluble in Triton X-100. It sediments in the native state as high molecular microfilamentous F-actin. Its identity was confirmed by immunfluorescence techniques using antibodies against liver actin (Nickola, personal communication). The most probable explanation for the role of the 43000 protein is, that this protein is a component of the subsurface core inside the membrane. It is unclear whether this subcellular structure is involved in the bile acid transport or not. The function of the 38000 and 32000 proteins remains obscure. Recently, an organic anion binding protein (bilitranslocase) with two subunits of 37000 and 35500 was described [32]. Both subunits could be extracted with EDTA from aceton powder prepared from liver plasma membranes. It might be possible that our 38000 and 32000 proteins are related to the above subunits.

All other labeled membrane proteins could be sufficiently separated by two-dimensional electrophoretic techniques. The existance of disulfide bonds between the labeled proteins was ruled out. (However, disulfide bonds exist between plasma membrane proteins as was demonstrated by the occurrence of off-diagonal spots in the Coomassie blue pattern after two-dimensional electrophoresis. In the study presented we were only interested in radioactively labeled proteins).

Comparable investigations were made by the group of G. Kurz with photoaffinity labels derived from taurocholic acid. These authors demonstrated that one of their labels did not markedly bind to membrane proteins in presence of a surplus of phalloidin and of antamanide [7]. In addition, their labels did not bind to membrane proteins of ascites hepatoma cells. A photoaffinity label, derived from the cyclopeptide antamanide (well known to block the uptake of phalloidin in liver cells) modified proteins with mole weights of 46000, 43000, 28000 and 60000 in liver plasma membranes (Nassal, unpublished data). This is of interest because antamanide probably interacts with the bile acid transporting channel. Recently, a 60000 protein was isolated from liver plasma membranes by affinity chromatography with bile acid labeled sepharose (Möller, unpublished data). A protein with the same mole weight was isolated by use of affinity chromatography with bilirubin and with BSP-labeled agarose [33]. 43000 and 54000 proteins [34] and a 55000 protein [35] may be responsible for binding of organic anions in liver cell plasma membranes. DIDS prevented binding of NAP-taurine to the 54000 and 43000 proteins [34] and also the covalent binding of [^{35}S]BTC. That means that binding sites for DIDS, for NAP-taurine and for BTC could be in near proximity or even identical.

Our present conclusion for the bile acid transport system of the liver cell is that the involved molecular structure consists of more than one native protein. It cannot be excluded that components of the above system have additional functions or cooperate alternatively with different transport systems. Probably, the 54000 protein is part of all anion transports, whereas the 50000 protein might be additionally needed for the uptake of bile acids.

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