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# 3′-ISOTHIOCYANATOBENZAMIDO[$^3$H]CHOLATE, A NEW AFFINITY LABEL FOR HEPATOCELLULAR MEMBRANE PROTEINS RESPONSIBLE FOR THE UPTAKE OF BOTH BILE ACIDS AND PHALLOIDIN

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**Substitution of the hydroxyl group on C7 of cholic acid by a benzamido group leads to a derivative with inhibiting quality for the inward transport of both bile acids and phallotoxins by isolated liver cells. The tritiated isothiocyanate derivative was prepared (3′-isothiocyanatobenzamido[$^3$H]cholate, [$^3$H]IBCA) with a specific activity of 70–80 mCi/mmol. The latter compound was used for affinity labeling of liver plasma membranes in order to detect chemically modified proteins involved in the transport of bile acids. [$^3$H]IBCA and the noncovalently binding analogs were recognized by the transport system; they inhibited the uptake of both [$^{14}$C]cholate and of demethyl[$^3$H]phalloin in vitro. Isothiocyanatobenzamidocholate (IBCA) was able to protect isolated hepatocytes against phalloidin. In isolated and purified plasma membranes prepared from liver cells [$^3$H]IBCA binds to saturable sites in an irreversible manner. Micromolar concentrations of unlabeled IBCA or millimolar concentrations of natural substrates prevented [$^3$H]IBCA binding in a concentration dependent manner; some other substrates of the transport system also protected liver membranes against chemical modification. Membranes from AS-3OD hepatoma cells, well known to transport neither bile acids nor phallotoxins, could not be labeled by [$^3$H]IBCA. The major targets of labeling in hepatocellular plasma membranes were polypeptides with molecular mass of 67, 60, 54, 50, and 37 kDa as shown by SDS-polyacrylamide gel electrophoresis (10% acrylamide). The 67 kDa protein could be found in the aqueous phase after phase separation in Triton X-114. The 54 kDa and 50 kDa proteins remained in the detergent phase and can therefore be regarded as integral membrane proteins.**

## Introduction

Phallotoxins, one type of toxic cyclopeptides of *Amanita phalloides* [1,2], can only be accumulated by intact hepatocytes [3–6] but not by other kinds of cells. In several previous kinetic studies [5–7] a strong parallelism of bile acid and phallotoxin transport was observed. Various compounds, known to inhibit the hepatocellular uptake of bile acids [8], also reduced the inward transport of demethyl[$^3$H]phalloin [8]. The same inhibitors protect liver cells against phalloidin [9–12]. Thus we concluded that phalloidin enters the hepatocytes by use of the same route normally reserved for the reabsorption of bile acids [11,12]. In order to elucidate the molecular structure of this transport system, several affinity labels for the bile acid

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Abbreviations: IBCA, 3′-isothiocyanatobenzamidocholate; [$^3$H]IBCA, 3′-isothiocyanatobenzamido[$^3$H]cholate; $H_2$DIDS, 4,4′-diisothiocyanato-1,2-diphenylethane-2,2′-disulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PCMB, *p*-hydroxymercuribenzoate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

transporter were synthesized earlier [13] and used for modification of membrane proteins [14]. However, chemical instability of brominated derivatives of bile acids as well as their relatively low affinity stimulated further activities to obtain a better tool for our problem. In the following the synthesis of a new derivative of cholate is described indicating some technical progress: isothiocyanatobenzamido[$^3$H]cholic acid ([$^3$H]IBCA) is a relatively stable reagent, applicable for several weeks after preparation; its affinity to liver plasma membranes is much higher than that of the brominated derivative.

## Materials and Methods

SDS and urea were purchased from Merck (Darmstadt, F.R.G.), PMSF, bovine serum albumin, TEMED, acrylamide, Triton X-114, *N,N'*-methylenebis(acrylamide) and Coomassie brilliant blue G from Serva (Heidelberg, F.R.G.). All other reagents were of the highest purity grade available commercially.

*Membrane preparation.* Plasma membranes were prepared from rat liver, and also from AS-30D ascites hepatoma cells according to Touster et al. (1970) [15] with some modifications [16].

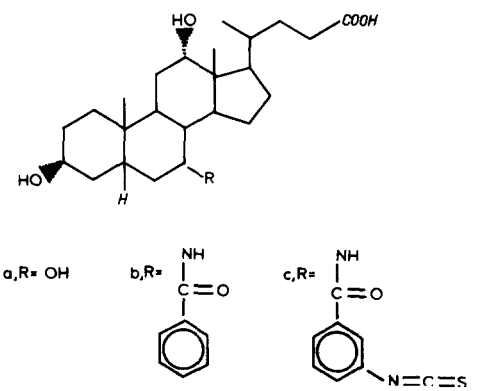
*Isolation of hepatocytes.* Hepatocytes were isolated according to Berry and Friend (1969) [17]. After an equilibrium period of 30 min in Tyrode buffer pH 7.4 at 37°C in $O_2/CO_2$ (95%/5%) atmosphere, about 85–90% of the cells were intact according to the exclusion of 0.2% Trypan blue.

*Uptake studies.* The uptake of demethyl[$^3$H]-phalloin (spec. act. 13.54 mCi/$\mu$mol) and [$^{14}$C]cholic acid (spec. act. 52 mCi/mmol) by isolated hepatocytes was estimated by a rapid centrifugation technique through silicon oil [18,9]. In all competition experiments hepatocytes were incubated with varying concentrations of either 7-benzamidocholic acid or unlabeled 3'-isothiocyanatobenzamidocholate 30 s prior to the addition of 1 $\mu$M [$^{14}$C]cholate plus 5 $\mu$M unlabeled cholate or of 6 $\mu$M phalloidin plus 1 $\mu$M demethyl[$^3$H]phalloin, respectively.

In experiments aimed at the determination of the irreversible part of inhibition, the hepatocytes were preincubated 5, 10, 20 and 40 min with varying concentrations of IBCA. After removal of the unbound label, the usual uptake studies were performed. The response of isolated hepatocytes to phalloidin was quantified by counting the number of affected cells as described earlier [9]. To test reversible effects on the phalloidin response of the bile acid derivatives, isolated hepatocytes ($2 \cdot 10^6$ cells/ml) were preincubated for 1 min with varying concentrations of benzamidocholic acid or IBCA at 37°C in Tyrode buffer (pH 7.4) prior to adding 10 $\mu$M phalloidin. After 20 min the amount of affected hepatocytes (cells with blebs) was determined. The irreversible part of inhibition was determined by preincubation for different periods with subsequent washing.

*Affinity labeling of isolated hepatocytes and of plasma membranes.* Isolated hepatocytes ($450 \cdot 10^6$ cells) suspended in Tyrode buffer (pH 7.4, 37°C) were incubated with 5 and 10 $\mu$M ($(8–16) \cdot 10^5$ dpm/ml) of [$^3$H]IBCA (spec. act. 70–80 mCi/mmol) during 40 min. After removal of the unbound label by washing with cold Tyrode buffer, the hepatocytes were subjected to plasma membrane preparation [15]. The homogenization was achieved by using the Standsted cell disrupter (Stansted Fluid Power, Ltd.).

In other experiments plasma membranes were isolated from whole liver and subsequently (1–2 mg/ml) incubated for 10–40 min with 2–200 $\mu$M ($(3–300) \cdot 10^5$ dpm/ml) of [$^3$H]IBCA in phosphate-buffered saline, Tyrode buffer or in 40 mM




Scheme I. Structural formulae. a, 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholan-24-oic acid: b, 3$\alpha$,12$\alpha$-dihydroxy-7$\beta$-benzamido-5$\beta$-cholan-24-oic acid; c, 3$\alpha$,12$\alpha$-dihydroxy-7$\beta$-(3'-isothiocyanato)benzamido-5$\beta$-cholan-24-oic acid (IBCA).

phosphate buffer plus 120 mM NaCl (pH 7.4) at 4°C, 20°C or 37°C, respectively. The reaction was stopped with 100 mM Tris-HCl (pH 7.4) at 0°C. The unbound label was removed by washing. The following proteinase inhibitors were added to the washing solution in order to prevent cleavage of proteins: PMSF (1 mM), benzamidine (1 mM), iodacetamide (1 mM), PCMB (0.01 mM). In some experiments 1% albumin was included in the buffer.

*Protein solubilization and analysis.* Protein was determined according to Lowry et al. (1951) [19], using bovine serum albumin as a standard. Phase separation of integral membrane proteins in Triton X-114 solution was performed as described by Bordier (1981) [20]. Lipid was extracted from plasma membranes using chloroform/ethanol (1 : 1, v/v). SDS gel electrophoresis was performed as described earlier [16]. Gels were cut in 2-mm slices. These slices were put into a scintillation vial, solubilized in 4 ml of Lipoluma/Lumasolve/$H_2O$, heated for 4 h at 50°C, and counted in a Packard scintillation counter. Fluorography of SDS slab gels was done according to Laskey and Mills (1975) [21].

*Synthesis of 7-benzamidocholic acid, IBCA and [$^3$H]IBCA.* The 7-keto derivative of cholic acid was synthesized according to the literature [22,23]. The preparation of its oxime followed the procedure of Redel et al. [24] with slight modifications: for purification the product was dissolved in methanol, and the solution was concentrated until a slight turbidity appeared. The product was then extracted with diethyl ether, the solution was dried and brought to dryness in the vacuum.

For the preparation of 3$\alpha$,12$\alpha$-dihydroxy-7$\beta$-aminocholanic acid 10 g of the oxime were dissolved in 200 ml of isoamyl alcohol, and the solution was heated to 100–110°C. 30 g of sodium were added in small portions during 4 h. After further heating for 1 h, the cooled solution was neutralized with 2.5 M $H_2SO_4$. After several hours the precipitate of sodium sulfate was filtered off and washed with dry ethanol. The ethanolic solution was brought to dryness under vacuum, and the material was again dissolved in ethanol. The solution was filtered and brought to dryness again. The sodium salt of the acid was then practically pure and was used for further synthesis. For analysis, a fraction was purified by chromatography over a column of XAD-2 (Serva Co.) in ethanol and water. The yield amounted to 71%, M.p.: 220°C (decomp.).

Analysis:Calculated:C: 67.10%, H: 9.38%, N: 3.26%.
Found :C: 67.68%, H: 9.25%, N: 2.98%.

3-Isothiocyanato- and 4-isothiocyanato-benzoyl chloride were prepared according to Stiefken [25]. For their reaction with the amine, 0.5 g of its sodium salt were suspended in 50 ml of tetrahydrofuran (dimethyl formamide in the case of the reaction with 4-isothiocyanatobenzoyl chloride) and 0.1 g of sodium bicarbonate in 2 ml of water were added. 0.3 g of the acid chlorides were added in portions during 30 min, and the mixture was stirred for a further 30 min. The mixture was then heated to the boiling point, and approx. 20 ml of saturated NaCl solution in water were added, until two phases were separating out. The organic solvent phase was brought to dryness under vacuum. The residue was extracted twice with 20 ml of boiling water and redissolved in methanol. The solution was filtered and brought to dryness again. The yield was 40%

Analysis:Calculated:C: 67.57%, H: 7.80%, N: 4.93%.
Found :C: 67.56%, H: 8.09%, N: 4.77%.

The IR spectrum showed the characteristic band for the isothiocyanate group at 2100 $cm^{-1}$ and bands at 1710 and 1640 $cm^{-1}$ for the benzamido group.

The synthesis of the radioactively labeled compound followed a different procedure. In the first step, 3-nitrobenzoyl chloride was coupled to the dihydroxyaminocholanic acid as described above for the 3-isothiocyanatobenzoyl chloride. The product was recrystallized from acetonitrile. The yield was 46.5%, m.p. 205°C.

Analysis:Calculated:C: 66.86%, H: 7.96%, N: 5.03%.
Found :C: 66.60%, H: 8.01%, N: 4.94%.

0.5 g of this compound were dissolved in 100 ml of dry acetone, then a solution of 1.0 g of aluminium *t*-butylate in 100 ml of dry benzene was slowly added with stirring. After boiling under reflux for 18 h, the solution was neutralized with 5% sulfuric acid, the organic layer was separated

and washed three times with water, twice with saturated $NaHCO_3$ solution, and once with water. After drying over sodium sulfate, the solution was brought to dryness under vacuum. The yellow oil crystallized after treatment with cyclohexane. The yield was 80.4%, m.p. 120–125°C. The presence of keto groups in the oxidation product was shown by infrared spectra, by a characteristic green colour reaction on thin-layer chromatograms in ethyl acetate/cyclohexane/acetic acid (50 : 10 : 5, v/v) with *m*-dinitrobenzene/KOH spray, and by analysis.

Analysis: Calculated: C: 67.37%, H: 7.29%, N: 5.06%.
Found : C: 67.38%, H: 7.42%, N: 5.00%.

80 mg of this product were dissolved in 4 ml of dry ethanol and partially reduced by the addition for 4 mg of sodium boro[$^3$H]hydride (100 mCi). After stirring for one hour, the solution was hydrogenated after addition of $PtO_2$ for 36 h. The solution was then evaporated to dryness, and the product was suspended in a small amount of 0.2 M HCl. It was applied to paper electrophoresis at pH 1.9, and the aromatic amine was thus purified and eluted from the paper with ethanol after autoradiography of the sheet for 12 h. The solution was again taken to dryness, and the product was dissolved in 3 ml dry dioxane. 0.1 ml of thiophosgene was added, and after 30 min the solvent was removed together with the excess of thiophosgene under vacuum. In larger batches of this synthesis, using labeled sodium borohydride, the product was shown to be identical with 3α,12α-dihydroxy-7β-(3′-isothiocyanato)benzamido-5β-cholanic acid by thin-layer chromatography, melting point, and infrared spectra.

## Results

*Inhibition by 7 benzamidocholate of cholic acid and phalloidin uptake*

7-Benzamidocholic acid was found to be a

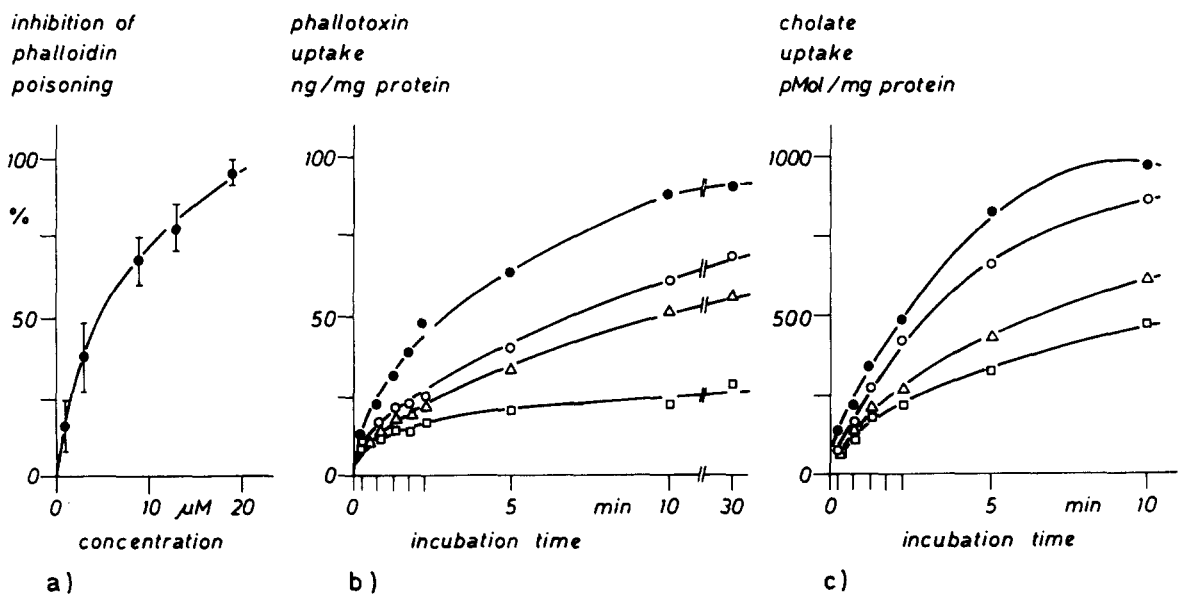



Fig. 1. Inhibition by 7-benzamidocholic acid of phalloidin response phallotoxin and cholate uptake. (a) Isolated hepatocytes ($2 \cdot 10^6$ cells/ml) were incubated in Tyrode buffer (pH 7.4, 37°C) with 2, 5, 9, 13, and 19 µM 7-benzamidocholic acid, 1 min prior to addition of 10 µM phalloidin. After 20 min incubation the number of cells with protrusions was estimated [9]. Shown is the mean of four experiments (error bars, standard deviations). (b) $2 \cdot 10^6$ hepatocytes/ml were incubated in Tyrode buffer with 2 µM (○——○), 9 µM (△——△) and 18 µM (□——□) 7-benzamidocholic acid for 30 s. The phallotoxin uptake was started by addition of 1 µM demethyl[$^3$H]phalloin plus 6 µM phalloidin. After 15, 45, 75, 105 s, 5, 10 and 30 min 100-µl aliquots were taken to measure the transport by a rapid centrifugation technique [18] (●——●), control). (c) Isolated hepatocytes were preincubated (30 s) with 2 µM (○——○), 9 µM (△——△) and 18 µM (□——□) 7-benzamidocholic acid prior to addition of 1 µM [$^{14}$C]cholic acid plus 5 µM cholate. The uptake was determined with the centrifugation technique after 15, 45, 75, 105 s, 5 and 10 min (●——●, control).

strong inhibitor of the phalloidin response of liver cells and of the uptake of both phallotoxins and cholate. The concentration of the label needed to produce 50% inhibition of the uptake of both substrates and of the phalloidin response was the lowest of all derivatives [13] tested in our laboratory as yet (Fig. 1).

The affinity of 7-benzamidocholic acid to the transporter was similar to that of cholic acid itself. The $IC_{50}$ values for 7-benzamidocholic acid, cholate and IBCA are $11 \pm 4$ μM, $8 \pm 1.7$ μM and $5 \pm 2$ μM, respectively ($IC_{50}$ values are defined as the concentration producing 50% of maximal inhibition of uptake of 1 μM [$^{14}$C]cholate). Insertion of the isothiocyanate group (NSC) into 7-benzamidocholic acid produced a suitable affinity label for studies on bile acid binding or transporting proteins. It must be proved whether the insertion of the NSC groups alters the properties of 7-benzamidocholic acid with respect to its inhibitory quality.

*Reversible and irreversible inhibition by IBCA of cholate and phallotoxin uptake and of the phalloidin response*

The uptake of both cholic acid and phallotoxin and also the phalloidin response were inhibited by reversibly and irreversibly bound IBCA in a concentration dependent manner. The degree of inhibition measured in the presence of IBCA exceeds that after removal of the unbound label by washing. After prolonged preincubation periods, the amount of irreversibly bound ICBA increased. The uptake of phallotoxin and cholate and the sensitivity of isolated hepatocytes to phalloidin decreased simultaneously (Fig. 2). IBCA inhibited the uptake of [$^{14}$C]cholic acid (1 μM) in a concentration-dependent manner. The concentration of IBCA needed for half maximal inhibition ($IC_{50}$) was lower than that of unlabeled cholate. These data suggest that cholic acid and its 3′-Isothiocyanatobenzamido derivative compete for the same binding site.

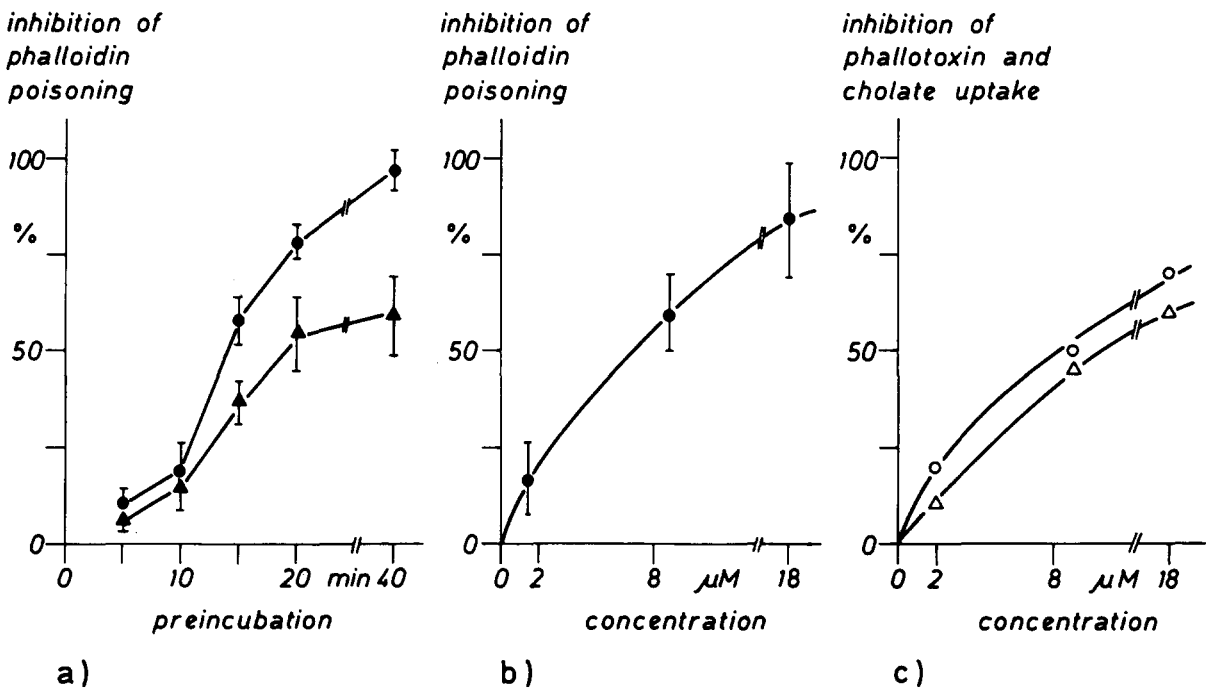



Fig. 2. Irreversible inhibition by IBCA of phalloidin response, phallotoxin and cholic acid uptake. (a and b) Irreversible inhibition of phalloidin response. Isolated hepatocytes ($2 \cdot 10^6$ cells/ml) were preincubated in Tyrode buffer (pH 7.4, 37°C) with 9 μM (●———●), 18 μM (▲———▲) IBCA for 5, 10, 15, 20, and 40 min (a) or with 2 μM, 9 μM, and 18 μM for 20 min (b). The unbound compound was removed by washing with fresh buffer. Thereafter 10 μM phalloidin was added. After 20 min the number of affected cells was counted. Shown is the mean of four experiments. Error bars, standard deviation. (c) Irreversible inhibition of phallotoxin and cholate uptake. Isolated hepatocytes were incubated for 15 min with 2 μM, 9 μM, and 18 μM IBCA before washing. The uptake of phallotoxin (△———△) and cholate (○———○) was measured for 10 min. For experimental details see legend of Fig. 1.

*Binding of [$^3$H]IBCA to isolated rat hepatocytes*

The uptake of cholic acid by hepatocytes is undoubtedly a saturable, energy dependent, and carrier-mediated process. For chemical modification of membrane proteins involved in the transport, intact hepatocytes were incubated with [$^3$H]IBCA. After 40 min incubation at 37°C with 5 or 10 $\mu$M [$^3$H]IBCA (concentrations previously found to inhibit cholic acid uptake to 30–60%), the unbound label was removed by washing. After preparation of plasma membranes from the cells, 30% of the initially added radioactivity was recovered in the homogenate; 1–2% thereof was bound to the plasma membrane. 42% of the radioactivity was detected in the $100\,000 \times g$ supernatant (cytosol) indicating that the compound was taken up by the hepatocytes like physiological bile acids. SDS-gel electrophoresis (10% acrylamide slab gels) of SDS-treated plasma membrane proteins revealed labeled polypeptides with apparent molecular masses of 67, 50, 54 (the latter two not always well resolved in 10% acrylamide gels), a 37 kDa and a 60 kDa protein (Fig. 3). About 40% of the total radioactivity was located in the region of 50–54 kDa proteins. Higher concentrations of [$^3$H]IBCA lead to increased labeling of the aforementioned polypeptides. However, labeling of additional polypeptides was not observed under the conditions described. Previous studies [26] on cholate binding to isolated plasma membranes revealed three binding sites, of which one high-affinity site was argued to be the putative carrier for hepatic cholic acid uptake. To elucidate molecular similarities of the cholic acid binding site and the cholic acid carrier, isolated plasma membranes from rat liver were radiolabeled with [$^3$H]IBCA.

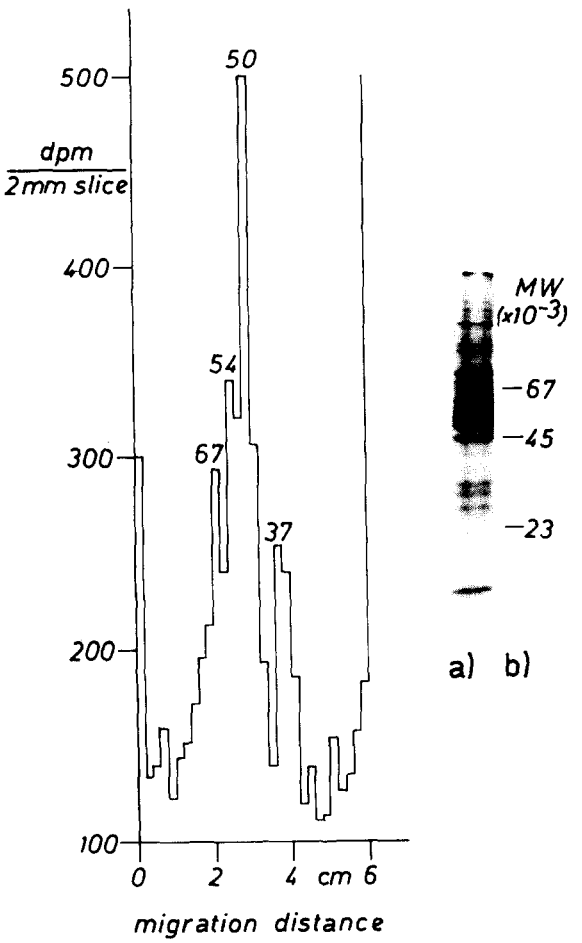



Fig. 3. Electrophoretogram and distribution of radioactivity in plasma membrane proteins prepared from [$^3$H]IBCA-labeled hepatocytes. Isolated hepatocytes were incubated with 5 $\mu$M [$^3$H]IBCA for 40 min. After removal of the unbound label plasma membranes were prepared and subjected to SDS-electrophoresis (10% acrylamide). The figure shows a Coomassie blue-stained electrophoretogram from isolated hepatocytes plasma membranes (a) as well as standard proteins: albumin 67 kDa, ovalbumin 45 kDa, trypsin 23 kDa (b). The distribution of $^3$H was estimated in SDS slab gels by slicing the gel (2 mm) and counting the activity in the gel discs.

*Binding of [$^3$H]IBCA to isolated plasma membranes*

Binding of [$^3$H]IBCA to plasma membrane proteins did not influence their electrophoretic mobility. The NCS group of IBCA reacts with $NH_2$ groups of polypeptides. The rate of thiocyanylation depends on the pH. Therefore special care was taken to do all experiments at pH 7.4 precisely.

Isolated plasma membrane vesicles were incubated at various concentrations of [$^3$H]IBCA for different incubation periods. After a standard washing procedure, 40–90% of the radioactivity remained membrane bound depending on the concentration of IBCA and the incubation time. The radioactivity recovered after gel electrophoresis (excluding the Bromophenol blue region) was in the range of 6–20%. That means about 80–90% of the $^3$H bound to the membrane was not covalently coupled.

About 50–60% of the radioactivity initially bound to the membranes could be extracted with

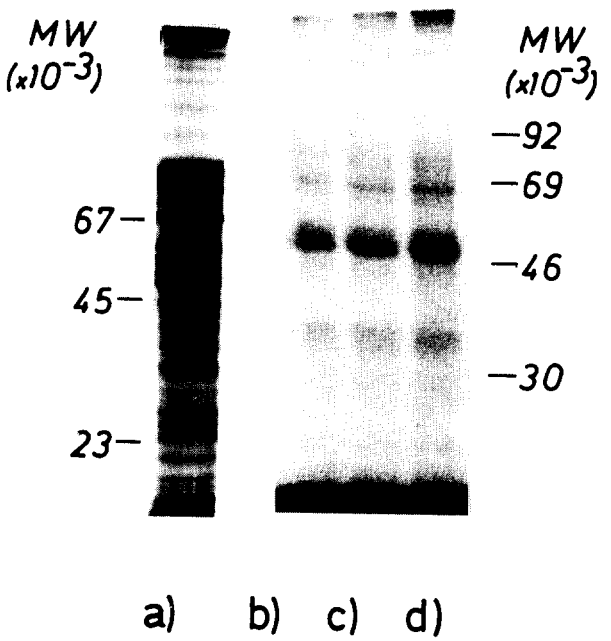



Fig. 4. Electrophoretogram and fluorogram of [$^3$H]IBCA-labeled plasma membrane proteins. Isolated plasma membranes in phosphate buffered saline (pH 7.4, 37°C) were incubated for 10, 20, and 40 min with 5 $\mu$M [$^3$H]IBCA. After removal of the unbound label SDS-gel electrophoresis (10% acrylamide) was performed. Lane a, Coomassie blue-stained SDS slab gel of isolated plasma membranes; lane b, fluorogram of [$^3$H]IBCA-labeled plasma membranes, 10 min incubation time; lane c, fluorogram, 20 min incubation time; lane d, fluorogram, 40 min incubation time.

chloroform/ethanol during 30 min. That means a remarkable part of the label was fixed by membrane lipids because of its hydrophobic nature. Addition of 1% albumin during the washing procedure also diminished the amount of the membrane bound label.

The amount of covalently fixed [$^3$H]IBCA increased during prolonged exposure. Fig. 4 shows the time-course of [$^3$H]IBCA binding. Furthermore final binding of [$^3$H]IBCA depended on the applied concentration. The major targets of the label are proteins with apparent molecular masses of 50, 54 (not always well resolved in 10% acrylamide gels), 67, 37 kDa as well as the 60 kDa protein, which was labeled to a not well reproducible extent. No additional labeled polypeptides were observed at label concentrations between 2 and 20 $\mu$M. In particular there was no difference between labeling of intact cells with subsequent separation of their plasma membranes and treatment of purified membranes prepared from whole liver (Fig. 5). At 200 $\mu$M [$^3$H]IBCA no addition incorporation of the radioligand into the 50–54 kDa proteins occurred, suggesting saturation of binding (Fig. 6).

As a control for specific labeling, plasma membranes isolated from AS-30D ascites hepatoma cells were treated with [$^3$H]IBCA. After removal of unbound label by our standard washing procedure, the total amount of radioactivity detected in the protein pellet resembled that of the radioactivity detected in hepatocyte plasma membranes. 40–60% of the radioactivity was associated with

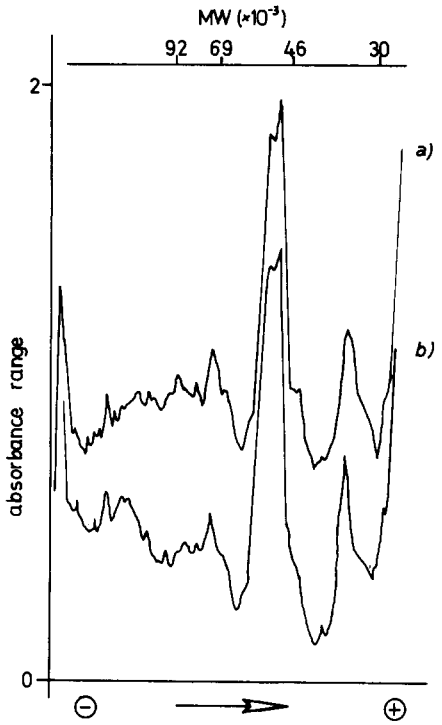



Fig. 5. Comparison of the labeling patterns after labeling of liver cells and after treatment of isolated liver plasma membranes with [$^3$H]IBCA. (a) Isolated hepatocytes were incubated in Tyrode buffer (pH 7.4, 37°C) with 10 $\mu$M [$^3$H]IBCA for 40 min. After removal of the unbound label their plasma membranes were prepared. Aliquots (100 $\mu$g) were separated by SDS-gel electrophoresis. Shown is a densitometric scan (at 540 nm) of the fluorography. (b) Isolated plasma membranes prepared from whole livers were incubated with [$^3$H]IBCA (10 $\mu$M) in Tyrode buffer (pH 7.4, 37°C) for 40 min. Thereafter unbound label was removed and the protein was separated by SDS gel electrophoresis. Shown is a densitometric scan (at 540 nm) of the fluorography.

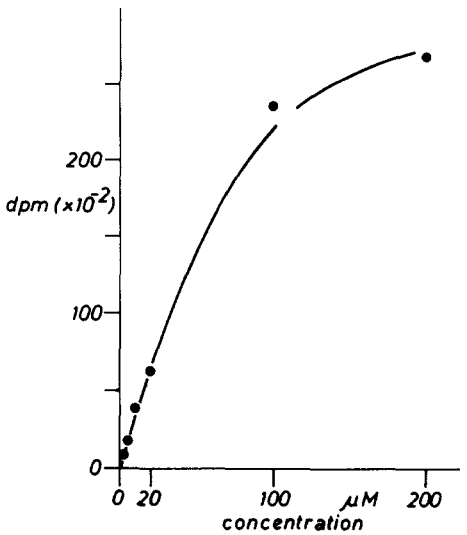



Fig. 6. Incorporation of [$^3$H]IBCA in to the 50–54 kDa proteins. Isolated plasma membranes (2 mg/ml) were incubated in phosphate-buffered saline with 2 μM, 5 μM, 10 μM, 20 μM, 100 μM, and 200 μM [$^3$H]IBCA for 20 min at 37°C. After removal of the unbound label aliquots (100 μg of protein) were subjected to SDS gel electrophoresis. The distribution of $^3$H was estimated by slicing the SDS slab gels in 2 mm discs and counting the radioactivity.

the protein pellet. Nevertheless, after SDS-electrophoresis no radioactivity peak was detected in the 50–54 kDa region. As shown in Fig. 7 the radioactivity background was high due to the hydrophobicity of the label.

The identity of the binding site for [$^3$H]IBCA with that for the unlabeled analog was established by pretreatment of membranes with unlabeled IBCA; this prevented the binding of the tritiated compound to the proteins in the 67, 60, 50–54 and 37 kDa region, Fig. 8A. In further studies labeling was carried out in the presence and in the absence of various substrates (taurocholic acid, phalloidin), competitive reversible inhibitors (Iopodate) [27], and irreversible inhibitors (DIDS) [12] of the transport system for bile acids. Micromolar concentration of DIDS and millimolar concentration of Iopodate and of bile acids (taurocholate or cholate) inhibited binding of [$^3$H]IBCA to the plasma membrane proteins (Fig. 8B). Preincubation of plasma membrane vesicles with phalloidin or addition of the substance together with [$^3$H]IBCA did not reduce the binding up to a 5000-fold excess of phalloidin. Higher concentrations could not be tested, because of solubility problems.

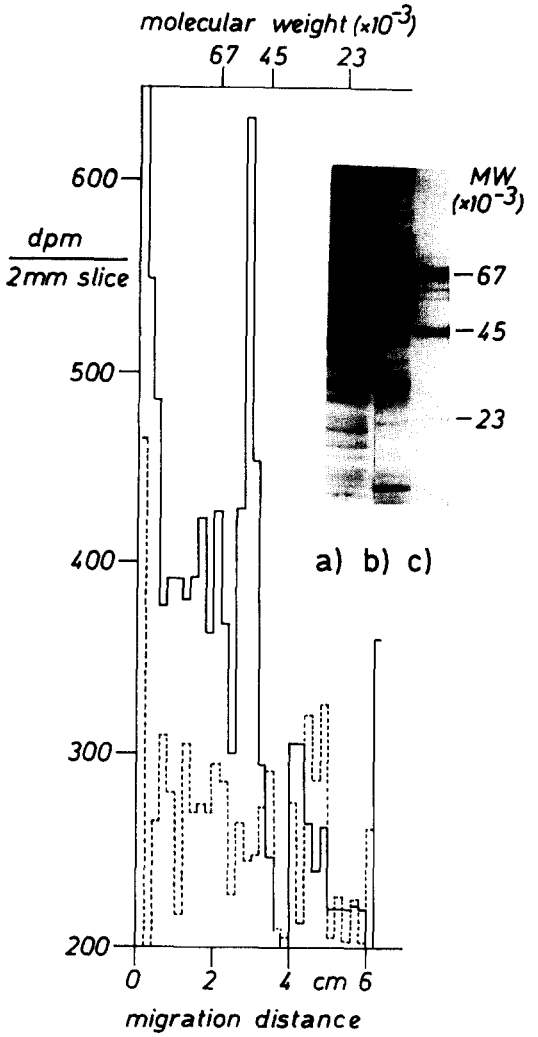



Fig. 7. Comparison of the labeling patterns in plasma membranes obtained from isolated liver cells and from AS-30D hepatocytes. Isolated plasma membranes from isolated hepatocytes (———) and from AS-30D ascites tumor cells ( — — —) were labeled with 5 μM [$^3$H]IBCA in phosphate buffered saline (37°C, pH 7.4) for 20 min. After removal of the unbound label, SDS gel electrophoresis was performed, SDS slab gels were sliced in 2 mm discs and the radioactivity was counted. Lane a, Coomassie blue-stained electrophoretogram of AS-30D ascites plasma membrane protein; lane b, Coomassie blue-stained electrophoretogram of liver plasma membrane proteins; lane c, standard proteins: bovine serum albumin 67 kDa, ovalbumin 45 kDa and trypsin 23 kDa.

The appearance of more than one labeled peak in SDS-electrophoretograms is unexplained. It

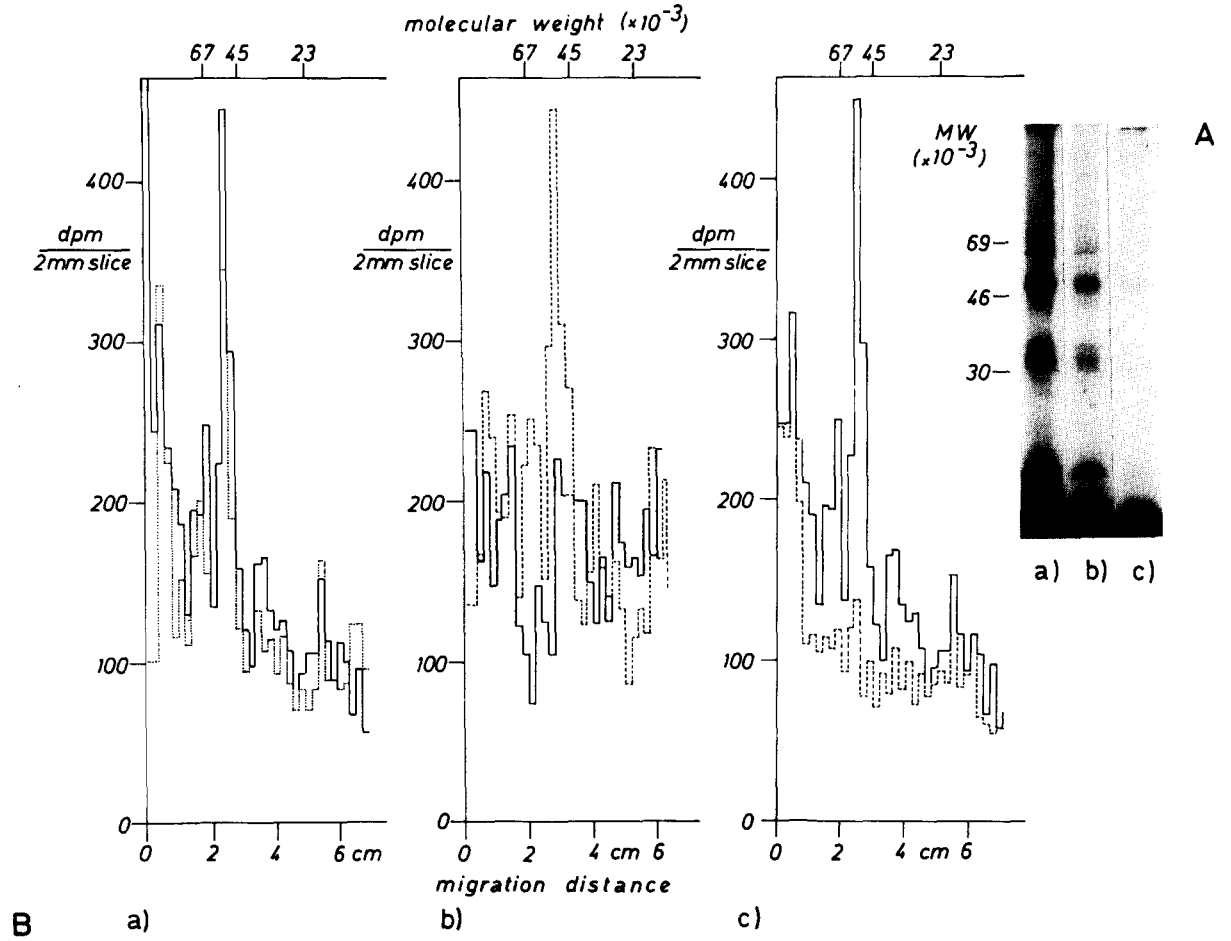



Fig. 8. (A) Protection by unlabeled IBCA of [$^3$H]IBCA binding to isolated liver plasma membranes. Isolated plasma membrane proteins (2 mg/ml) were incubated in 40 mM phosphate buffer plus 120 mM NaCl (pH 7.4) without (a) or with 50 $\mu$M (b) and 500 $\mu$M (c) IBCA before adding 4 $\mu$M [$^3$H]IBCA. After 20 min the reaction was stopped and the unbound label was removed by washing. Aliquots were subjected to SDS gel electrophoresis and fluorographic analysis of labeled proteins. (B) Protection of DIDS, taurocholate and Iopodate of [$^3$H]IBCA binding to isolated liver plasma membranes. (a) Isolated plasma membranes (2 mg/ml) were incubated without (———) or with 1 mM (- - - - -) taurocholate before adding 4 $\mu$M [$^3$H]IBCA. For further processing see legend to part (A) above. (b) Isolated plasma membranes were incubated without (- - - - -) or with 1 mM Iopodate (———) before adding 4 $\mu$M [$^3$H]IBCA. (c) Plasma membranes were incubated without (———) or with 200 $\mu$M DIDS (- - - - -) before adding 4 $\mu$M [$^3$H]IBCA. Aliquots were subjected to SDS gel electrophoresis. The distribution of [$^3$H]IBCA was determined by slicing the gels and counting the radioactivity.

might be caused by the action of proteases. However, the addition of different protease inhibitors (PMSF, benzamidine, soybean trypsin inhibitor, PCMB, EDTA) during the plasma membrane preparation and during labeling did not reduce the number of labeled polypeptides (data not shown).

Membranes are composed of two different kinds of proteins, namely integral ones, characterized by their hydrophobicity, and membrane associated ones which are more hydrophilic [29]. Integral proteins interact with detergents and can be separated from water soluble ones by phase separation techniques using Triton X-114 [20]. The 67 kDa labeled protein was found almost quantitatively in the aqueous phase suggesting its hydrophilic nature. The labeled proteins in the 54–50 kDa region remained in the detergent phase, pointing to their integral character (Fig. 9).

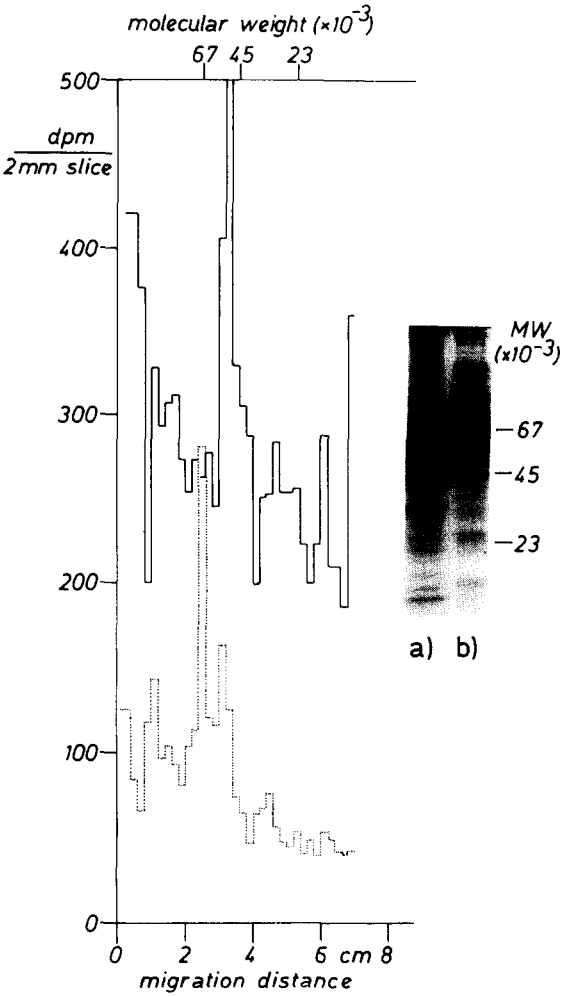



Fig. 9. Triton X-114 phase separation. [$^3$H]IBCA-labeled liver plasma membranes were subjected to phase separation in Triton X-114. Hydrophobic proteins remained in the detergent phase, hydrophilic ones in the water phase. Both kinds of proteins were separated by SDS gel electrophoresis. Lane a, Coomassie blue-stained gel of hydrophobic proteins; lane b, Coomassie blue-stained gel of hydrophilic proteins. ———, distribution of [$^3$H]IBCA-labeled hydrophobic proteins in gel slices; — — —, distribution of [$^3$H]IBCA-labeled hydrophilic proteins in gel slices.

Solubilization of labeled membrane proteins with Triton X-100, Nonidet P-40 or taurodeoxycholate resulted in the recovery of the labeled proteins in the supernatant. Labeling of the 100 000 × $g$ supernatant after solubilization with Nonidet P-40 lead to the chemical modification of the same polypeptides (Fig. 10).

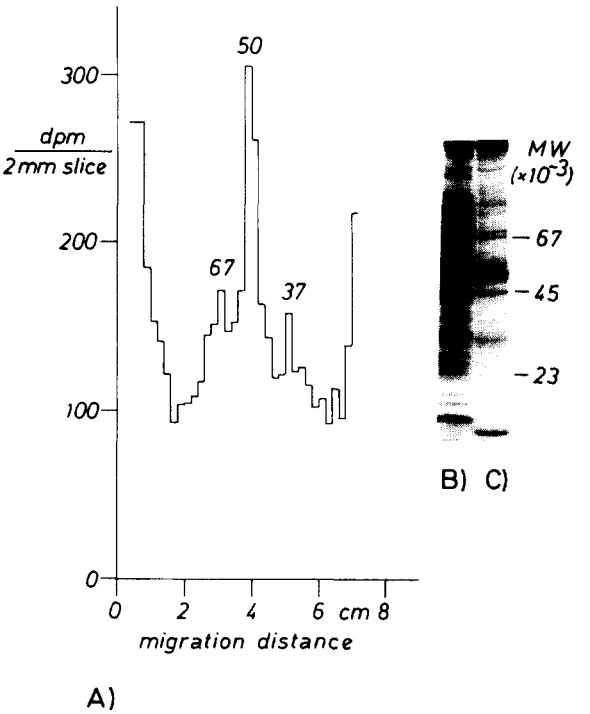



Fig. 10. Labeling of Nonidet P-40 solubilized plasma membrane proteins. Isolated plasma membranes in phosphate-buffered saline were solubilized in 1% Nonidet P-40. The proteins remaining in the supernatant after high-speed centrifugation were labeled with [$^3$H]IBCA for 20 min at 37°C. After removal of unbound label by dialysis, SDS electrophoresis was performed. The labeled polypeptides were detected by slicing SDS slab gels. (A) Distribution of Nonidet P-40 solubilized [$^3$H]IBCA-labeled proteins in gel slices. Lane B, Coomassie blue-stained SDS gel of Nonidet P-40 solubilized proteins; lane C: Coomassie blue-stained SDS gel of proteins unsoluble in Nonidet P-40.

## Discussion

In previous papers we compared the binding of [$^3$H]$H_2$DIDS and of brominated taurocholate derivatives to liver plasma membranes [16,14]. Surprisingly both labels bound to identical polypeptides as judged by SDS-electrophoresis. Because of the chemical instability of the brominated taurocholate a more stable label was synthesized. Besides of much better handling of the new label it's inhibitory potency for phalloidin uptake and response as well as for cholic acid uptake, was increased 4-fold. Furthermore the new reagent

could be synthezized with a 500-fold higher specific radioactivity.

Comparison of the results presented with those gained in earlier studies reveals the following similarities: All labels included the new one, bind to membrane polypeptides with the same molecular masses (67, 60, 54, 50 kDa). Labeling could be prevented by preincubation with the corresponding unlabeled substance. Protection of the above polypeptides could also be achieved with antagonists or with natural substrates of the carrier system. The protection by 1 mM taurocholic acid (Fig. 8Ba) however was negligible. It must be taken into account that bile acids solubilize membranes when applied in concentrations above 1 mM. It would be therefore nonsensical to test such concentrations in our experiments. DIDS (Fig. 8Bc) and the unlabeled analogue of [$^3$H]IBCA (Fig. 8A), both irreversibly binding compounds, occupied nearly all binding sites for [$^3$H]IBCA.

We expected that phalloidin also protects the carrier system in liver cell membranes against [$^3$H]IBCA labeling as shown by Kurz with a photoaffinity label derived from taurocholic acid [30]. A series of earlier kinetic data support the idea that phalloidin uptake by liver cells is mediated by one of the bile salt transporters [5,6]. Cholate is a competitive inhibitor of the phallotoxin uptake [7]. However, the affinity of the acidic substrates to the transporter is higher than that of phalloidin. Low concentrations of cholate inhibit the uptake of demethylphalloin ($K_I = 8$ $\mu$M) completely whereas a 200-fold excess of phalloidin reduces the cholate uptake to a small extend only [5]. We don't know which groups of phalloidin are recognized by the transporter. These are probably more hydrophobic than the reactive groups which are able to couple with isothiocyanates. It is therefore possible that neutral cyclopeptides of the phallotoxin type protect transporter proteins against certain labels, whereas others do not. Isothiocyanate groups prefer amino groups of membrane proteins, whereas the reactive groups activated by photolysis may bind to any part of the molecule near to the label. As expected transport deficient hepatoma cells were not labeled specifically. The high background labeling seen, was due to the hydrophobic character of [$^3$H]IBCA which penetrates into the lipid phase of the membrane.

Labeling of isolated hepatocytes with subsequent preparation of plasma membrane produced the same modification pattern of membrane proteins as the one observed in experiments with plasma membrane isolated prior to incubation with [$^3$H]IBCA. The label itself was taken up by hepatocytes (radioactivity in the $100\,000 \times g$ supernatant = cytosol) like a physiological bile salt. Only 1–2% [$^3$H]IBCA was irreversibly bound by the plasma membrane fraction prepared from labeled hepatocytes.

[$^3$H]IBCA labeling depended on time and concentration. Above 200 $\mu$M unspecific labeling of additional polypeptides occurred. The multiplicity of labeled polypeptides is an unresolved phenomenon. The 67 kDa protein is water soluble and can be extracted together with the 37 kDa protein by EDTA [16]. Furthermore it separates in the aqueous phase in Triton X-114 solutions. The localization (at the external or at the cytoplasmic site of the membrane) is unknown as yet.

Labeling studies with plasma membrane vesicles at 4°C (no/or markedly reduced transport of bile acids [28]) resulted in reduced binding of [$^3$H]IBCA to the 67 and 37 kDa protein suggesting a cytoplasmatic localization. Further studies are needed with a 100% right-site-out population of plasma membrane vesicles or with hepatocytes at 4°C.

The 60, 54 and 50 kDa proteins are integral ones, whether they are transmembrane ones is still unclear.

One possibility of the multiplicity of modified proteins is a proteolytic cleavage of proteins during the preparation. We used a combination of protease inhibitors during some plasma membrane preparations without reducing the number of radioactive peaks. But this does not rule out any proteolytic cleavage. Studies with still other (photo)affinity labels on the same biological system are necessary for further investigation of structural properties of the transporter proteins. Nevertheless it is obvious that all investigations reported up to now gave similar results with different labels. At present it is unclear, whether the identified proteins belong to different transport systems; it is also possible that they are subunits of a complex system consisting of channel proteins and binding proteins on the surface. These facts challenge further studies with other (photo)affinity labels as well as with chemical crosslinkers.

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