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INVESTIGATIONS ON THE HEPATIC UPTAKE SYSTEMS FOR ORGANIC CATIONS WITH A PHOTOAFFINITY PROBE OF PROCAINAMIDE ETHOBROMIDE

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Abstract—Azido procainamide methoiodide (APM), a photolabile derivative of the transport model compound procainamide ethobromide (PAEB), shows a close resemblance to PAEB from a physicochemical point of view. Like PAEB it is effectively taken up by the liver and excreted into bile. Kinetics of the uptake of APM in isolated hepatocytes revealed that in addition to a non-saturable process, two saturable uptake systems are involved (K_{m1} = 3 μM, V_{max1}) = 80 pmol/min/10^6 cells, K_{m2} = 100 μM, V_{max2} = 130 pmol/min × 10^6 cells). The uptake rate of APM was inhibited markedly in the presence of other organic cations. Organic anions and uncharged compounds generally had no inhibitory effect on the APM uptake. These results support the theory that there is a separate hepatic uptake system for organic cations like APM. Photoaffinity labeling of intact hepatocytes as well as plasma membrane sub-fractions enriched with sinusoidal domains disclosed two major binding polypeptides with apparent M_r of 48,000 and 72,000. Such labeling patterns were not observed in membranes from hepatoma cells that are deficient in organic solute uptake. Differential photoaffinity labeling with other cationic compounds such as tributylmethyl ammonium and *d*-tubocurarine reduced the incorporation of APM in these polypeptides. The 48- and 72-kDa proteins might be involved in carrier-mediated transport of type I organic cations at the hepatic uptake level.

Procainamide ethobromide (PAEB‡) and its *N*-acetyl derivative APAEB are the classic model compounds used in hepato-biliary transport of organic cations. The combined data of numerous studies on PAEB and APAEB transport demonstrate that the hepatic uptake of these organic cations occurs by a carrier-mediated process [1–6]. In general, the transport pathway for relatively small (type I) organic cations is thought to be different from that for uncharged compounds and organic anions [2, 7–9]. Type I organic cations such as PAEB display a clear separation of the cationic group from the more hydrophobic ring structures in the molecule. In addition, the occurrence of hepatic uptake systems with broad overlapping substrate specificity accommodating amfipathic compounds irrespective of charge was suggested [7, 10, 11]. Results from experiments using photoaffinity probes of model compounds of various charge seem to support the concept of such an aspecific uptake system in the liver, since in principle they all label polypeptides in the same relative molecular mass range [10, 12–19]. Alternatively a family of closely related but different proteins might be involved [7, 20].

At present only limited data are available on the identity of carrier proteins for organic cations in the liver. Results from kinetic and photoaffinity labeling studies with the bulky organic cation *N*-propyldeoxyajmalinium indicate that this quaternary ammonium compound is substrate for the type II organic cation uptake system and/or for the above-mentioned multispecific uptake system in the liver [10, 16]. Kinetic data indicate that type I cations like PAEB are transported by a transport system that differs from the pathway for bulky organic (type II) cations that have the onium group included in ring structures [3, 7, 21–23]. In order to identify the binding polypeptides for the type I organic cations, we synthesized azido procainamide methoiodide (APM), a photolabile derivative of PAEB [24] (see Fig. 1). In this study the hepatic uptake of APM was characterized in the intact liver and isolated hepatocytes. The carrier system for organic cations was investigated further performing photoaffinity labeling of isolated hepatocytes and liver plasma membrane fractions.

MATERIALS AND METHODS

Chemicals. 4-Azido-*N*-[2-(diethyl[^{3}H]methylammonium)ethyl]benzamide iodide ([^{3}H]APM) (sp. act. 4.3 Ci/mmol) and unlabeled APM were synthesized as described previously [24]. Dibromosulphthalein was from SERB (Paris, France). Quinidine sulphate was from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hexafluronium bromide was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Indocyanine green was from Hynson, Westcott and Dunning (Baltimore, MD, U.S.A.).

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‡ Abbreviations: APM, azido procainamide methoiodide; PAEB, procainamide ethobromide; APAEB, *N*-acetylprocainamide ethobromide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

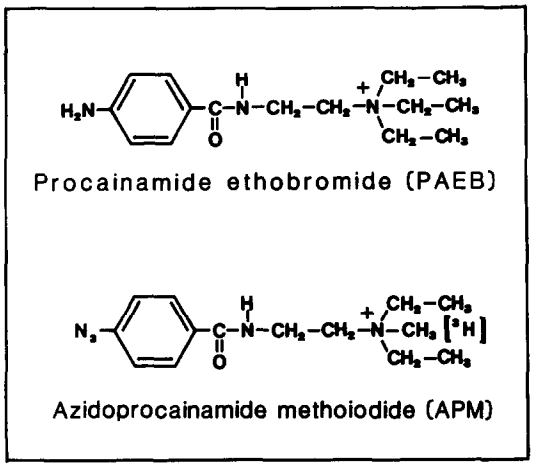

Fig. 1. Comparative, chemical structures of PAEB and APM.

Iodipamide was obtained from Schering AG (Berlin, F.R.G.). Sodium taurocholate was from Fluka AG (Buchs, Switzerland). Tributylmethylammonium iodide was synthesized as described by Neef *et al.* [25]. Sodium cholate was from Calbiochem (San Diego, CA, U.S.A.). K-Strophanthoside was obtained from Serva (Heidelberg, F.R.G.). Ouabain and *para*-aminohippuric acid were from Merck (Darmstadt, F.R.G.). *N*-Propyldeoxyajmalinium chloride was synthesized as described previously [23]. PAEB was from Squibb Inc. (Princeton, NJ, U.S.A). D-Tubocurarine was obtained from Burroughs Wellcome (London, U.K.). Bovine serum albumin (Boseral™) and vecuronium were from Organon Teknika (Oss, The Netherlands). All other reagents were of the best quality available from commercial sources.

Animals. Male Wistar rats (Centraal Dieren Laboratorium, University of Groningen) weighing 250–300 g and maintained on a standard rat diet and tap water *ad lib.* were used for kinetic studies in perfused liver and isolated hepatocytes. The animals were fasted for 16 hr before the experiment and anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) before surgery. Male Wistar rats (Tierzuchtanstalt Jautz, Hannover, F.R.G.) maintained under comparable conditions were used for the preparation of hepatocytes and sinusoidal membranes used in the photoaffinity labeling experiments.

Protein binding. Protein binding was determined at four concentrations (1, 10, 100 and 1000 μM) in a Krebs-bicarbonate buffer (pH 7.4) containing 1% bovine serum albumin by ultrafiltration using the Amicon MPS-1 filtration system (Amicon Corp., Danvers, MA, U.S.A.) at 37°.

Partition coefficient. In order to establish the relative lipophilicity of APM the octanol/Krebs partition coefficient was determined as described by Neef and Meijer [6], with some slight modifications. The partition system consisted of 5.0 mL *n*-octanol-1 and 5.0 mL Krebs solution from which sodium bicarbonate was omitted and the pH was adjusted to 7.4 with sodium hydroxide. [^{3}H]APM (100 nmol; total activity 0.8 μCi) was added and the two phases were mixed on a whirlmixer for 1 min. The mixture was shaken on a rotary tumbler (40 rpm) for 30 min at 20°. The layers were separated by centrifuging and 0.5-mL samples were submitted to liquid scintillation counting.

Isolated liver perfusions. Isolated perfused liver experiments were performed using the recirculating perfusion technique as described by Meijer *et al.* [26] with some slight modifications. The perfusion medium consisted of a Krebs-bicarbonate buffer supplemented with 10 mM glucose and 1% bovine serum albumin. A volume of perfusion medium of 100 mL was used in all experiments. The perfusate flow was adjusted to 3.5 mL/min × g liver. Perfusions were performed over a 1-hr period. Plasma samples (250 μL) were taken at different times and bile was collected during intervals of 5 or 10 min. At the end of the experiments the liver was homogenized in a saline solution and samples were submitted to liquid scintillation counting. Liver content at various time points was calculated by subtracting from the administered dose the amount in the perfusate plus the amount excreted in bile. Substances functioning as potential uptake inhibitors were added as a bolus injection 1 min prior to the addition of APM.

Isolation of hepatocytes. Liver cells from the uptake experiments were prepared by a modification of the original procedure of Berry and Friend [27], as described in a previous paper [22]. Cell viability, as assessed by Trypan blue exclusion, ranged from 88 to 97% with mean viability being 93%.

Uptake experiments in isolated hepatocytes. To analyse the uptake of APM in isolated rat hepatocytes, a suspension of liver cells (2.5–3 × 10^6 cells/mL) in Krebs-bicarbonate buffer, supplemented with 1% albumin, was preincubated for 30 min under carbogen gassing in a shaking water bath (37°) [22]. After preincubation the experiment was started by adding 75 μL of a solution containing both the labeled and the unlabeled APM, to yield a final volume of 3.0 mL. The uptake was determined over a concentration range of 1–1200 μM. Aliquots of 200 μL were withdrawn at 1, 2, 3, 4 and 5 min after addition of APM and the uptake was terminated by rapid centrifugation through a silicon oil layer. Subsequently, the amount of radioactivity in the cell fraction was estimated by liquid scintillation counting. Uptake was also measured in hepatoma ascites AS30D cells. Temperature dependency of uptake was studied by measuring initial uptake rate at various temperatures ranging from 17° to 42°. After preincubation the cells were placed in a water bath at varying temperatures. The cells were allowed to equilibrate for 3 min to the new temperature before addition of APM (10 μM). The results were plotted according to Arrhenius to calculate the apparent activation energy.

Preparation of plasma membrane subfractions from rat liver. Plasma membrane fractions enriched with sinusoidal surfaces were isolated from the liver as described previously [28]. This preparation should be considered as a partially purified sinusoidal membrane fraction since like all preparations described until now it contains small amounts of

microsomal membranes (endoplasmic reticulum and Golgi derived) as well as canalicular vesicles. The latter contamination, however, is very small since the major 100-kDa bile acid binding polypeptide [10, 13, 23] is not labeled significantly in this preparation.

Photoaffinity labeling of membrane sub-fractions of rat liver, isolated hepatocytes and AS30D hepatoma cells was performed as described previously [15, 28].

In brief, the procedure was carried out in a thermostated rotating Duran glass cuvette by irradiation at 300 nm for 10 min after preincubation with the photolabile organic cation. Membrane fractions were resuspended in 10 mM HEPES buffer pH 7.4. Aliquots of 200 μL (0.5 mg of protein) were preincubated with the photoaffinity probes for 5–10 min in the dark. The suspension was then pipetted in the cylindrical Duran-50-photolysis cell that was thermostatically controlled at 30° in the Photoreactor 400 (Gräntzel, Karlsruhe, Germany). Isolated cells ($5–20 \times 10^6$ per experiment) were incubated in polycarbonat tubes in 1–10 mL of the cell medium and photolysis was performed in a Rayonet-RPR-100 Photoreactor (The Southern New England Ultraviolet Co., Hamden, CT, U.S.A.), equipped with 16 RPR-3000 A° lamps. For differential photoaffinity labeling experiments, identical procedures were used but irradiation occurred in the presence of 200 μM of various inhibitors. After irradiation the suspensions were removed and cells were centrifuged at 1000 *g*. The supernatant with unbound radioactivity was removed and the cells were resuspended in 2.0 mL of the cell medium and centrifuged again. This wash procedure was repeated twice. The membrane fractions were washed with HEPES buffer and centrifugated for 30 min at 47,000 *g*.

Preparation of protein samples following photoaffinity labeling. One hundred microlitres of the protein-containing solutions were treated with 400 μL of methanol and after rigorous mixing, centrifuged at 12,000 *g*. One hundred microlitres of chloroform was then added, and the centrifugation was repeated after mixing. After addition of 300 μL of water and rigorous mixing, the mixture was again centrifuged and the supernatant was removed. The lower layers including the interphase were mixed with 300 μL methanol and then, after virorous mixing, centrifuged. The precipitate containing the proteins was collected and dried. Before performing separation procedures the proteins were dissolved in appropriate buffer solutions. For preparation of a total membrane fraction from the hepatocytes the washed cells were subsequently permeabilized by repeated freezing in liquid nitrogen and thawing. The cells were then centrifuged in a 5534 rotor of a Sorvall ultracentrifuge for 30 min and the pellet was delipidated with methanol and chloroform before application to the gel as described above [16, 23].

Photoaffinity labeling, SDS–PAGE and detection of radioactivity. Photoaffinity labeling was performed both on intact isolated rat hepatocytes and on plasma membrane fractions of normal hepatocytes and AS30D hepatoma cells. After photoaffinity labeling at 300 nm, shown to photolyse APM [24], samples were subjected to discontinuous SDS–PAGE [29] using vertical slab gels ($200 \times 140 \times 2.8$ mm). After electrophoresis the gels were fixed and stained with Coomassie brilliant blue as described previously [29] and the stained polypeptides were detected by scanning the gels at 575 nm with a densitometer (Vitatron, Dieren, The Netherlands). The distribution of the radioactivity was determined by liquid scintillation counting after cutting the gel into slices of 2 mm thickness and solubilization in an appropriate scintillation cocktail. The relative molecular masses of the labeled polypeptides were estimated by comparison with proteins of known relative molecular mass, using as references soybean trypsin inhibitor (M_r 20,100), carbonic anhydrase (M_r 30,000), ovalbumin (M_r 43,000), bovine serum albumin (M_r 67,000) and phosphorylase (M_r 94,000).

RESULTS

Physicochemical parameters

The unbound fraction of APM in the Krebs-bicarbonate buffer containing 1% bovine serum albumin was 0.85 over the concentration range studied (1–1000 μM). A low affinity high capacity type of binding might be involved [21, 30]. The small protein binding was later disregarded since it was unlikely to influence the results of the uptake experiments to a significant degree. The lipophilicity of APM, expressed as the octanol/Krebs partition coefficient at pH 7.4, was 0.135 ± 0.003 ($N = 3$).

Transport in isolated perfused liver

To characterize APM as a photolabile model compound for hepatic organic cation transport, we first investigated the hepato-biliary transport of APM in the intact organ. The results from the isolated perfused liver experiments (Fig. 2) show that APM (100 nmol) was effectively taken up by the liver with an initial clearance of 22.7 ± 0.7 mL/min. Of radioactivity, 49.4 ± 6.0% was excreted in bile within 60 min, partly as metabolites. Since this study deals primarily with the hepatic uptake mechanism of organic cations, no further efforts were made to determine the identity of these metabolites. When a bolus injection of the organic cation tributylmethylammonium iodide (1 μmol) was given shortly prior to the addition of APM, the hepato-biliary transport of APM was reduced substantially. Initial clearance decreased to 2.9 ± 0.5 mL/min, the maximum biliary excretion rate decreased from 2.5 to 0.9 nmol/min and the time point of maximum biliary excretion rate shifted from 13 to 27 min.

Uptake into isolated hepatocytes

To study the mechanism by which APM is transported into the liver cell, uptake experiments in isolated hepatocytes were performed. Uptake was linear over the first 5 min of incubation. From the slope of the lines, determined by least-squares analysis, the initial uptake velocities V_0 for the different concentrations were calculated. When these initial uptake velocities were plotted against the corresponding APM concentrations, a curve–linear pattern was observed (Fig. 3). From the slope of the linear part of the curve, indicative of a non-saturable

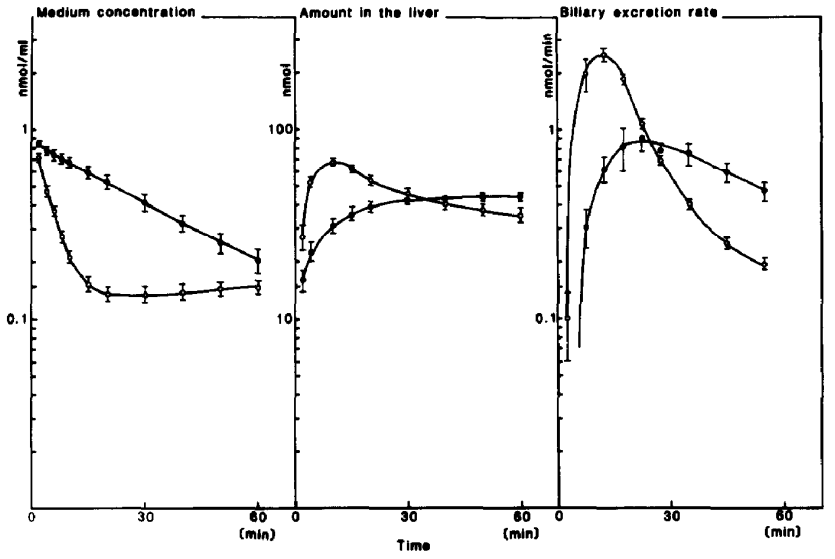

Fig. 2. Kinetics of APM in the isolated perfused rat liver after a bolus injection of 0.1 μmol (open circles), and after addition of a bolus injection of tributylmethylammonium iodide (1.0 μmol) immediately prior to the injection of APM (closed circles). Concentrations in perfusate, amount in the liver and biliary excretion rate are indicated. The curves depicted are the means ± SEM of three experiments.

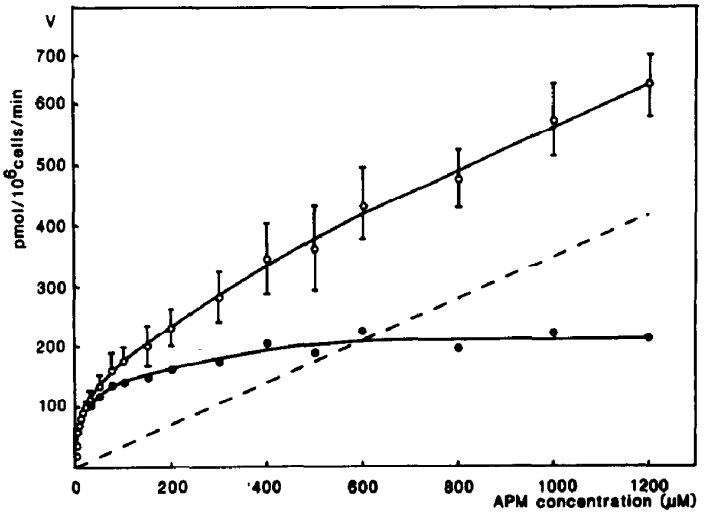

Fig. 3. Plot of initial uptake velocity (V_o) vs substrate concentration (S_o) in isolated hepatocytes (open circles). Each point represents the mean ± SEM of four experiments. The saturable part of the uptake (closed circles) was obtained after subtracting the contribution of the linear portion (broken line) from the total uptake.

process [31], a rate constant k of 0.35 pmol/10^6 cells/min/μM was calculated. Subtracting the non-saturable part from the total uptake yielded the initial uptake velocity of the saturable component for each concentration (Fig. 3). A V/S versus V plot [32] of these data yielded a biphasic curve (Fig. 4A). Because no cooperativity could be demonstrated, the shape of the curve may be described either by two different carrier systems or a single carrier- or channel-like membrane translocator with multiple sites of different affinities. This curve could be described by two saturable transport processes with $V_{max} = 80$ pmol/min × 10^6 cells, $K_{m1} = 3$ μM and $V_{max2} = 130$ pmol/min × 10^6 cells, $K_{m2} = 100$ μM. Uptake into AS30D hepatoma cells was minimal at all of the concentrations that were also employed in the normal hepatocytes. Uptake rate in normal hepatocytes was strongly temperature dependent.

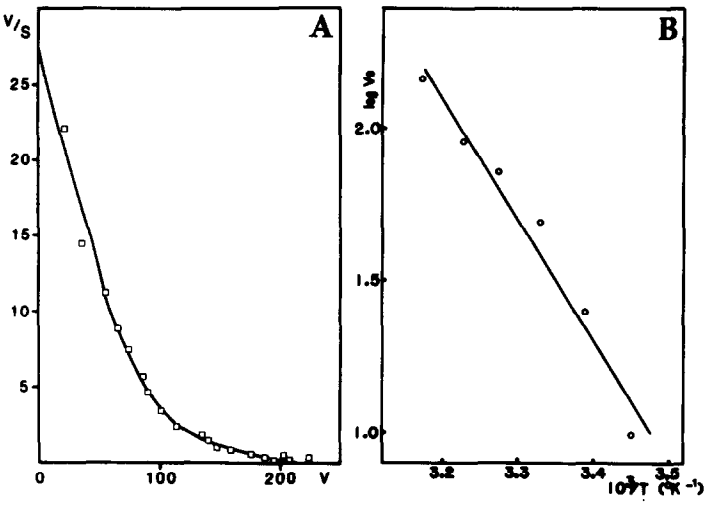

Fig. 4. (A) V/S vs V plot to determine the kinetic parameters of the saturable part of the APM uptake in isolated rat hepatocytes (open squares). The incubation buffer contained, among the other constituents, 112 mM NaCl and 25 mM NaHCO. Kinetic analysis revealed two uptake systems with the apparent kinetic constants $K_{m1} = 3\,\mu M$, $V_{max1} = 80$ pmol/min $\times 10^6$ cells; $K_{m2} = 100\,\mu M$, $V_{max2} = 130$ pmol/min $\times 10^6$ cells. (B) Temperature dependency of APM uptake in hepatocytes. Arrhenius plot of initial uptake rate of APM at 10 μM versus $1/T$. The slope indicates an apparent activation energy of 77 kJ/mol.

From the Arrhenius plot an apparent activation energy of 77 kJ/mol could be calculated (see Fig. 4B).

Effect of other substrates on the uptake of APM

In order to elaborate structural specificity of the hepatic uptake systems for APM, also in relation to the data reported on its parent compound PAEB, the influence of several classes of hepatic model compounds on the uptake of APM (30 μM) was determined at an inhibitor/substrate ratio of 3.3 (Fig. 5). At the concentration of 30 μM, the high affinity system contributes to about 70% of the saturable uptake while the non-saturable part accounts for less than 10%. The results show that the parent compound PAEB decreased the initial uptake velocity by 58%. The other monovalent and bivalent organic cations studied also inhibited substantially the uptake of APM into the liver cells. The anionic compounds studied, including the bile salts, did not influence the uptake of APM, with the exception of indocyanine green (79% inhibition). Since multiple transport systems seem to be involved and classical kinetic analysis with amfipathic organic compounds often fails to provide definite clues, in the present study no attempts were made to determine the type of inhibition. However, studies with tributyl methyl ammonium at least showed mutual competitive inhibition [33]. The initial uptake velocity was not affected by the uncharged compounds studied.

Photoaffinity labeling

Photoaffinity labeling of the liver plasma membrane fraction disclosed at least two major bands in which the radioactivity was incorporated (Fig. 6). The apparent M_r of these bands were 33,000 and 48,000. The broad band with apparent M_r of 48,000 may contain more than one polypeptide (see Fig. 7). In addition, a more variable, relatively small band was consistently identified at an apparent M_r of 72,000. Photoaffinity labeling of a membrane fraction, prepared from previously photolabeled hepatocytes, also revealed two bands with apparent M_r of 48,000 and 72,000 (Figs 7 and 8). The major M_r 33,000 polypeptide in these preparations was not identified in the plasma membrane preparation enriched in sinusoidal fragments, indicating that the polypeptide with apparent M_r of 33,000 that is labeled in intact hepatocytes is probably of intracellular rather than of sinusoidal origin or alternatively, represents a loosely associated membrane protein that was lost during isolation procedures. No consistent labeling of polypeptides was found in the 100–200-kDa region. In this range, potential carrier proteins for organic cations from the canalicular domain of the plasma membrane have been identified in experiments with bulky organic cations in canalicular membrane fractions [10, 16]. The distinct labeling patterns as observed in membranes from normal rat hepatocytes were virtually absent in hepatoma cells (see Fig. 7).

Differential photoaffinity labeling of intact hepatocytes in the presence of tributylmethylammonium or *d*-tubocurarine revealed a decreased incorporation of the photolabel into the polypeptides of M_r 48,000 and 72,000 both in plasma membranes and hepatocyte total membrane fraction (Fig. 8). The two cationic agents also partly reduced the incorporation of APM into other polypeptides (see Fig. 8).

With regard to the inhibition experiments with the intact hepatocytes, the decreased incorporation

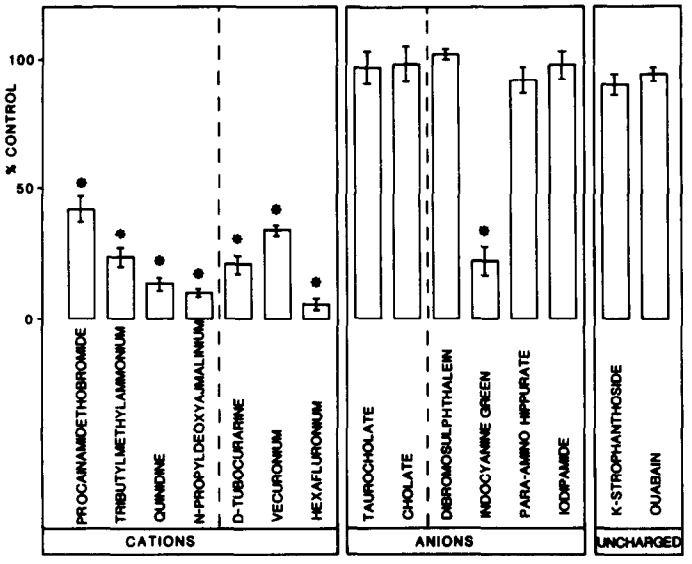

Fig. 5. Effect of substrates of different charge on the initial uptake of APM in isolated rat hepatocytes. APM concentration was 30 μM. Substrate concentration was 100 μM. Results are the means ± SEM of three or four experiments.

by these competing cations in the 48- and 72-kDa as well as in the other protein peaks of the total membrane fraction could be due to direct inhibition of the labeling of general binding proteins in the membranes and/or to a general decrease in the uptake of APM during irradiation, by which the labeling of intracellular proteins is also affected indirectly. In addition, the APM probe may label potential carrier proteins and other polypeptides from the inside of the plasma membrane so that through uptake inhibition a general decrease in labeling is observed. Nevertheless, the high degree labeling of the 48- and 72-kDa proteins combined with the much lower degree of labeling of the 33- and 100-kDa proteins in the plasma membrane preparation is compatible with a preferential labeling of the sinusoidal domain of the plasma membrane.

DISCUSSION

In order to identify transport polypeptides for organic cations in the hepatocyte plasma membrane, we recently synthesized APM [24]. This photolabile derivative of procainamide closely resembles in its structure the classic model compound for organic cations, PAEB (see Fig. 1). APM appears to be a suitable photoaffinity probe, since efficient photolysis occurred at a wavelength of 300 nm [24].

Physiocochemical parameters like albumin binding and lipophilicity were determined in order to establish whether APM corresponds with its parent compounds PAEB and APAEB with regard to these parameters, that can have a marked influence on transport characteristics [6, 7, 9, 34]. Albumin binding of APM was rather low (unbound fraction > 0.85) and resembles in this respect the reported values for APAEB (0.86–0.94) [4, 6, 35] and PAEB (0.98) [21]. Lipophilicity, expressed as the octanol/Krebs partition coefficient also yielded a value in the same range as PAEB and APAEB [6]. Additionally, APM properly fits the relationship between lipophilicity and relative molecular mass, as described by Neef and Maijer [6]. These results demonstrate that APM besides its structural resemblance also parallels its parent compounds PAEB and APAEB from a physicochemical point of view.

To be useful as a model compound for the hepatic transport of organic cations, adequate hepato-biliary transport of APM should occur in the intact organ. The results from the experiments using the isolated perfused liver demonstrate that APM is indeed effectively taken up by the liver and subsequently excreted in bile, partly in metabolized form. Liver/plasma and bile/liver concentration ratios exceed 20 and match the values obtained for PAEB and APAEB [4, 9, 21]. The results point to the occurrence of two concentrative steps in the hepato-biliary transport of APM. In addition, the inhibitory effect of tributylmethylammonium, a relatively low molecular mass cation that has been shown to be taken up in liver by saturable mechanism(s) [25, 31, 36], indicates that APM uptake occurs by carrier-mediated mechanisms.

In isolated hepatocytes the uptake of APM was studied in more detail. The results showed that APM is taken up by saturable and non-saturable processes. Kinetic analysis of the saturable part of the uptake revealed the existence of a high and a low affinity transport system, with capacities in the same order of magnitude. Eaton and Klaassen [5] reported saturable uptake of PAEB in isolated hepatocytes with $K_m = 54\ \mu M$ and $V_{max} = 130$ pmol/min × mg protein. They analysed the data in terms of a single

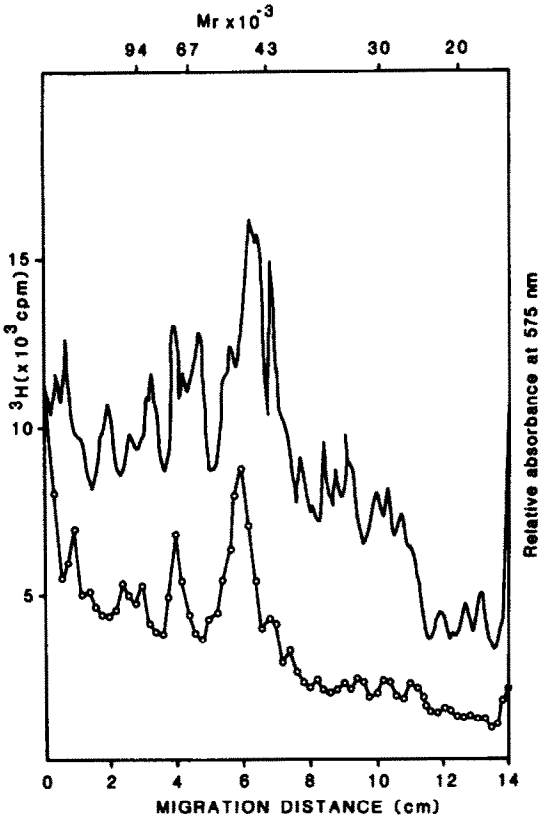

Fig. 6. Distribution of radioactivity after SDS–PAGE of plasma membrane sub-fraction enriched in sinusoidal elements from rat liver following photoaffinity labeling with APM (open circles). One milligram protein of plasma membrane sub-fraction was photolabeled with 5.8 μmol/L (5 μCi) of [^{3}H]APM. For all other conditions, see the legend to Fig. 5. Relative absorbance at 575 nM is indicated in the upper curve (solid line).

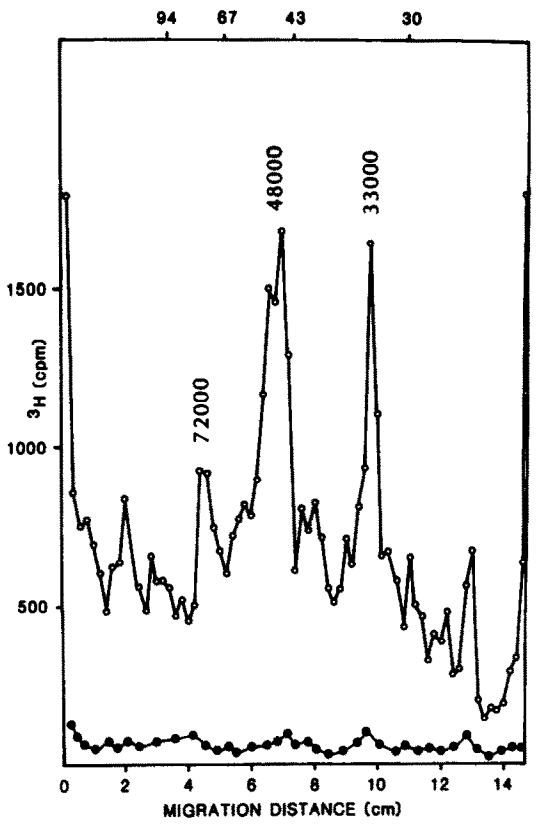

Fig. 7. Distribution of radioactivity after SDS–PAGE of the total membrane fraction obtained after photoaffinity labeling of isolated hepatocytes with APM. Cells (5 × 10^6/mL) were photolabeled at 300 nm and 30° with 4.7 μmol/L (20 μCi) of [^{3}H]APM. Total acrylamide concentration of the gel was 9% at a ratio of acrylamide/bisacrylamide of 97.2/2.8. The lower curve (closed circles) indicates incorporation of radioactivity in the total membrane fraction of irradiated hepatoma (AS30D) cells that lack organic cation transport.

transport system that exhibits features of the low affinity system encountered here, since in our cell preparation 10^6 cells equal 1.6 mg protein. Calculation of the clearance in isolated hepatocytes and in the intact organ, as described by Blom *et al.* [37], shows that the clearance in cells (0.0223 mL/min × 10^6 cells) corresponds with the clearance in the isolated perfused liver (0.0246 mL/min × 10^6 cells), indicating that the transport function is well preserved in isolated hepatocytes. The uptake process for APM showed a distinct temperature dependency. The activation energy of 77 kJ/mol is fully compatible with a non-passive uptake system as reported for various substrates [22, 33].

The data so far mentioned indicate that the photolabile PAEB derivative APM is taken up by the liver via carrier-mediated mechanisms. As a crucial criterion for carrier-mediated transport, the inhibition by structurally related compounds further strengthens this conclusion. All the organic cations tested markedly inhibited the uptake of APM into the hepatocytes at an inhibitor/substrate ratio of 3.3. The uptake inhibition by indocyanine green is likely to be caused by the zwitter-ionic character of this compound. The positively charged groups in the molecule may lead to binding with the presumed uptake system for APM [7]. With respect to the specificity of the uptake system for APM, the lack of effect of the other organic anions and uncharged model compounds supports the theory that there is a separate transport system for relatively small organic cations (the so called type I system [7, 38]).

Photoaffinity labeling of isolated hepatocytes with APM disclosed two major polypeptides in which the radioactivity was incorporated, with apparent M_r of 33,000 and 48,000. Photoaffinity labeling of a hepatocyte membrane fraction enriched with sinusoidal elements indicated that the M_r 33,000 polypeptide is not of sinusoidal origin or is only loosely associated with the membranes. Previously it has been shown with photoaffinity probes of bile acids and type II organic cations, that the polypeptides with apparent M_r of 33,000 are often of a mitochondrial origin [12, 23]. This protein band

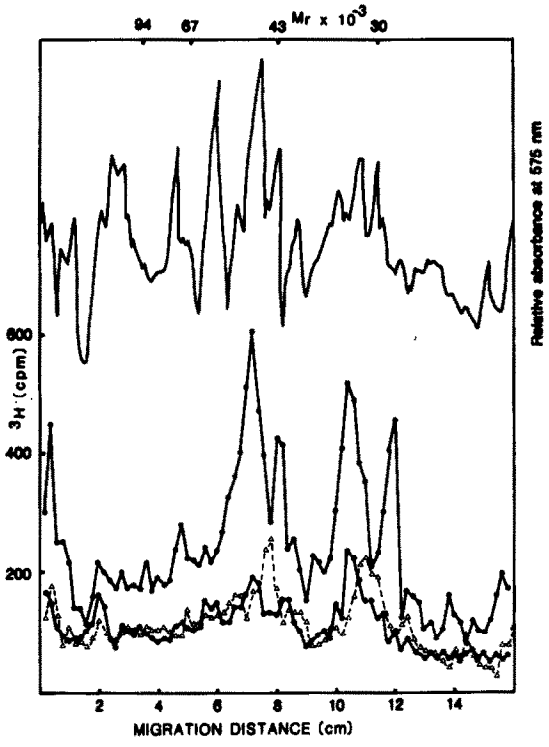

Fig. 8. Distribution of radioactivity after SDS–PAGE of the total membrane fraction obtained after photoaffinity labeling of isolated hepatocytes with APM. Cells (5×10^6/mL) were photolabeled with 4.7 μmol/L (20 μCi) of [^{3}H]-APM. Relative absorbance is indicated in the upper curve (solid line). For all other conditions see legend to Fig. 5. (●) No addition; (○) in presence of 400 μmol/L *d*-tubucurarine, (△) in presence of 400 μmol/L tributylmethylammonium iodide.

may represent a polypeptide for binding of amipathic organic compounds including cations. Alternatively, a more specific organic cation binder may be present in the inner mitochondrial membranes that may facilitate permeation into the organelle. Indeed, mitochondria have been shown to concentrate organic cations as a consequence of their transmembrane potential [39, 40]. Carrier-mediated uptake of organic cations in mitochondria might be involved [41].

Photoaffinity labeling of the sinusoidal membrane fraction also revealed binding polypeptides with apparent M_r of 48,000 and 72,000. The polypeptide with apparent M_r of 72,000 was also labeled in the intact hepatocytes, but in this preparation the labeling was more variable and less pronounced compared with other bands of activity. Differential photoaffinity labeling in intact hepatocytes in the presence of inhibitors of APM uptake showed a decreased incorporation in the M_r 48,000 and 72,000 polypeptides. No clear labeling occurred in these peaks in membranes from hepatoma AS30D cells that were also shown to be completely deficient in organic cation uptake (present study, Ref. 23). The combined data indicate that the M_r 48,000 and 72,000 proteins are involved in binding of APM to the sinusoidal membranes. It remains to be established whether these polypeptides represent the uptake systems that have been demonstrated in isolated hepatocytes. If such a relationship existed, the difference in the extent of labeling between the 48,000 and 72,000 polypeptides could be related to the different affinities of the two systems. Since in the present photoaffinity labeling studies low substrate concentrations are required, labeling of the high affinity system is favoured. Yet, differences in the quantity of both transport systems in the hepatocyte membrane could also explain the difference in the extent of labeling.

Binding polypeptides with an apparent M_r of 48,000 have been identified with several photolabile derivatives of substances that are taken up by the liver by carrier-mediated mechanisms, such as bile salts [10, 13, 18, 28], ouabain [19], *N*-propyl deoxyajmalinium chloride [10, 16, 23] and several mushroom poisons [15, 18]. For the above-mentioned substances mutual uptake inhibition has been demonstrated in isolated hepatocytes. The combined data of photoaffinity labeling and kinetic experiments with these agents suggest the existence of a "multispecific" transport system with broad substrate specificity in the hepatocyte membrane [10, 11, 19, 40]. Yet, the results from the kinetic studies reported here indicate that APM uptake occurs by a transport system distinct from this multispecific transport system. An important aspect here is that bile salts do not affect uptake of type I cations such as PAEB, APAEB or APM (Fig. 5) or only do so in extremely high concentrations [5, 35], whereas both categories of compounds label polypeptides in the 48-kDa region. Also, bile salts have only very modest effects on APM labeling of liver plasma membranes and *vice versa* [16]. In contrast, bile salts strongly reduced the hepatic uptake rate of type II organic cations [22, 38, 40, 42] as well as photoaffinity labeling with type II probes [10, 16, 23] and mutual effects on these parameters were observed [16, 23].

Consequently, the kinetic data from the interaction experiments in combination with the observed labeling with APM of a polypeptide with apparent M_r of 48,000 suggests the existence of at least two transport polypeptides in the M_r range of 48,000 in the sinusoidal membrane. Autoradiographic data obtained after photoaffinity labeling with several other photolabile derivatives also pointed to protein heterogeneity in the range of M_r 48,000 [10, 13, 16, 23]. Also, in the range of M_r 55,000 where bile acid and BSP binding polypeptides have been identified, protein heterogeneity has been postulated [20]. The functional significance of this protein heterogeneity is not known at present. The different polypeptides probably originate from an archetypal transport protein that has evolved into several more specific transport proteins. Slight modifications of the recognition area could change the specificity, whereas the relative molecular mass would be virtually identical. For other proteins, such as the cytochrome P450 system, the existence of a series of highly comparable proteins has been described [43]. These proteins differ in substrate

specificity and have similar relative molecular masses but different amino acid sequences. This marked enzyme heterogeneity can be seen as a prerequisite to cope with the huge variety of xenobiotics to which the organism is exposed. Considering the analogy with the cytochrome P450 system, the existence of a group of transport polypeptides with apparent M_r of about 48,000 in the sinusoidal domain of the plasma membrane of the hepatocyte is conceivable. Such proteins could be involved in the effective hepatic extraction of a wide variety of substances from the blood.

In conclusion, the combined data show that with APM, which resembles PAEB from a physicochemical and kinetical point of view, at least two polypeptides with apparent M_r of 48,000 and 72,000 are photolabeled in plasma membrane fractions of the liver. These polypeptides are apparently involved in the binding of APM to the hepatocyte membranes and further studies are required to substantiate that they are involved in the hepatic uptake of type I organic catiions.

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