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TRANSPORT OF ORGANIC ANIONS INTO LIVER CELLS AND BILE

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Abstract—The distribution of [^{14}C]*para*-acetylaminohippuric acid ([^{14}C]PAAH) and [^{35}S]sulfobromophthalein ([^{35}S]BSP) among plasma, liver cells and bile of rats was measured under steady-state conditions. The electrical potential difference (PD) was measured across the sinusoidal membrane of the liver cell and between plasma and bile in the common duct. A PD of 40.3 ± 1.0 mV (mean ± S.E.M.) was recorded across the sinusoidal membrane (cell negative with respect to plasma). The PD between plasma and bile was 3.9 ± 0.5 mV (bile negative with respect to plasma). The electrical potentials, considered together with the concentrations of PAAH and BSP in plasma, liver cells and bile, indicate that these organic anions are actively transported into liver cells across the sinusoidal membrane. The concentrations of PAAH and BSP in liver cells and bile suggest that they are transferred across the canalicular membrane by active transport processes. When the plasma concentration of PAAH was increased, the bile/liver cell ratio of PAAH reached an asymptote, suggesting that the transport of PAAH from liver cells into bile is a carrier-mediated system which can be saturated. An infusion of sodium taurocholate, which stimulated bile flow and increased the excretion of BSP in bile, did not influence the biliary excretion of PAAH. The transport of PAAH from liver cells into bile appears to act as a 'sink', thereby maintaining a low liver cell PAAH concentration. The membrane transport system for the uptake and excretion of PAAH may be relevant to the biliary excretion of other exogenous and endogenous organic anions. The results of this study also demonstrate the importance of considering electrical potentials when studying the distribution of a charged compound in the hepato-biliary system.

The transport of organic anions from plasma into bile involves at least two membrane transfer steps. Anions are first taken up by the liver cell across the sinusoidal membrane and then excreted into bile across the canalicular membrane. The biliary concentration of many organic anions greatly exceeds their plasma concentration, which suggests that one or both of these membrane transfer steps is an energy-requiring transport process. A basic fact of active transport processes is that the substance being transferred moves against an electrochemical gradient.

There is evidence that the transfer from plasma into the liver cell of substances such as sulfobromophthalein (BSP) and indocyanine green (ICG) is the result of binding to an intracellular cytoplasmic protein (ligandin) [1]. In bile such substances are also associated with mixed micelles which decrease their effective concentration and promote further biliary excretion [2]. Thus, for some compounds the process of biliary excretion appears to be equilabrative as well as intrinsically concentrative.

Nevertheless, active transport mechanisms may be important for the biliary excretion of smaller organic anions that are not bound extensively to proteins or associated with mixed micelles. In this study the electrical potential differences as well as the concentration gradients for the organic anions, *para*-acetylaminohippuric acid (PAAH) and BSP, were measured between plasma and liver cells and liver cells and bile under conditions of normal and bile acid stimulated bile flow. The results demonstrate that these organic anions are transferred between plasma and bile against electrochemical gradients.

METHODS

Materials. Sodium taurocholate was obtained from Steraloids, Pawling, NY, and contained no other bile acids, as determined by thin-layer chromatography. [^{35}S]Sulfobromophthalein ([^{35}S]BSP, 2.16 μCi/μmole) was purchased from Amersham-Searle, Arlington Heights, IL. PAAH was prepared by acetylation of *para*-aminohippuric acid (PAH) (Eastman Chemical Co., Rochester, NY) [3]. *Para*-acetylaminohippuric acid [*carboxy*-^{14}C] ([^{14}C]PAAH) was prepared by a modification of this method. Ninety micrograms of [^{14}C]PAH (10.8 μCi/μmole, New England Nuclear, Boston, MA) were dissolved in 1.0 ml water, and 10 μl of acetic anhydride were added to this solution. The mixture was allowed to stand at room temperature for 60 min. The aqueous solution was then evaporated under nitrogen and the residue was redissolved in 0.9% NaCl for administration to rats. [^{14}C]PAAH was determined to greater than 99 per cent pure by paper electrophoresis (0.25 M formate–acetate buffer, pH 1.9, at

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1000 V for 2.5 hr) and radioscan. When [^{14}C]PAAH was given to rats, the radioactive compound excreted in bile had the same electrophoretic mobility as the administered substance. Thus, it would appear that PAAH was not metabolized prior to excretion in the bile.

Animals and surgical procedures. Male Sprague–Dawley rats (180–230 g) were maintained on laboratory chow and water *ad lib*. Each animal was fasted for 12–18 hr but was allowed free access to water until the beginning of the experiment. All animals were anesthetized with sodium pentobarbital (45 mg/kg, i.p.); supplemental doses were given as needed. A cannula was inserted into the trachea and a functional nephrectomy was achieved by ligation of the renal pedicles. A cannula in the right femoral vein facilitated the placement of an agar bridge into the femoral vein. Solutions were infused via a cannula in the left femoral vein at a rate of 0.02 ml/min (Harvard Apparatus Small Animal Constant Infusion Pump). The bile duct was exposed through a midline abdominal incision and cannulated with polyethylene tubing. Bile was collected in pipettes calibrated in 0.01 ml volumes. Rectal temperature was maintained between 37° and 38° by a negative feedback circuit between a heating pad beneath the rat and a thermistor in a rectal temperature probe.

Experimental design. Constant biliary excretion of [^{35}S]BSP was achieved by an i.v. infusion at a rate of 0.02 μCi/min for 30 min. Constant biliary excretion of [^{14}C]PAAH was achieved by the i.v. infusion of 0.025 μCi/min for 5 min followed by an infusion of 0.002 μCi/min for 25 min. Each animal received one of the following treatments: (1) infusion of [^{14}C]PAAH or [^{35}S]BSP; (2) [^{14}C]PAAH or [^{35}S]BSP plus sodium taurocholate (0.3 μmole/min); or (3) [^{14}C]PAAH plus carrier PAAH in doses ranging from 0.25 to 2.0 mg/kg/min. During constant biliary excretion of either [^{14}C]PAAH or [^{35}S]BSP, bile was collected for 15 min, electrical potentials were recorded (see below), and the animals were exsanguinated from the abdominal aorta. The liver was immediately removed and weighed. A 220-mg sample of liver was used for radioactivity determination, and 1 g was homogenized in 10 ml of 0.1 M phosphate buffer, pH 7.4, for protein binding studies.

Determination of radioactivity. Bile, plasma and liver samples were mixed with 1.0 ml of 1.0 M piperidine and digested at 60° for 24 hr. An aliquot of this digest was transferred to a glass vial containing 16 ml of scintillation medium. The scintillation medium consisted of 10 ml toluene containing 6 g of 2,5-diphenyloxazole (PPO) per liter of toluene, and 6 ml of 2-ethoxyethanol. Radioactivity was measured with a liquid scintillation spectrometer (Isocap 399, Nuclear Chicago Corp.) utilizing a channel ratio method for single labeled samples.

Total liver radioactivity was corrected for protein binding (see below). Unbound radioactivity contained in the extracellular space and the residual bile volume of the liver were subtracted from the total liver radioactivity to determine the intracellular isotope concentration. A liver extracellular space of 11 per cent [4] and a residual bile volume of 0.005 ml/g liver [5] were employed in these calculations. Radioactivity in plasma was corrected for protein binding so that concentrations of PAAH and BSP are expressed as unbound compound. PAAH and BSP concentrations were converted to μmoles/ml by using the known specific activity of the material administered. Separation of BSP metabolites in liver and bile was not attempted.

Protein binding. Binding of [^{14}C]PAAH and [^{35}S]BSP to plasma and liver proteins was measured by equilibrium dialysis for 24 hr at room temperature. One milliliter of either plasma or liver homogenate was placed on one side of a lucite dialysis chamber. One milliliter of 0.1 M phosphate buffer, pH 7.4, was placed on the other side of the chamber. The solutions were separated by a cellophane dialysis membrane. After rotating the dialysis chambers gently for 24 hr, the solutions on both sides of the dialysis membrane were removed for measurement of radioactivity. Binding of [^{14}C]-PAAH to plasma and liver proteins was 36 per cent and 48 per cent, respectively. Binding of [^{35}S]BSP to plasma and liver proteins was 97 per cent and 93 per cent, respectively.

Measurement of plasma–bile potential difference (PD). The PD between plasma and bile was measured between two calomel half-cells as modified from the method of London *et al.* [6]. One half-cell was connected by an agar–0.1 N NaCl bridge to the femoral vein. The other calomel half-cell was connected by a similar agar bridge to a vial of 3 M KCl into which the bile duct cannula was immersed. Both calomel electrodes were connected to an electrometer with a 25 mV full scale deflection. The PD was read directly from the electrometer.

Measurement of liver cell PD. The PD between extracellular fluid and the liver cells was measured as described previously in our laboratory [7]. Liver cell potentials were recorded with stationary KCl-filled glass micro-electrodes (5–20 M ohms). A base-line was recorded with the micro-electrode immersed into a pool of saline (within a lucite ring) that was in contact with the liver surface. The extracellular fluid of the rat was grounded through a silver chloride electrode placed in the peritoneal cavity. The glass micro-electrode was then advanced into the liver tissue. A sharp, negative deflection of greater than −30 mV which could be maintained for at least 5 sec, with a sharp return to base-line on withdrawal of the electrode, was the criterion required for an adequate intracellular potential recording. Approximately 10–15 potentials were recorded from each animal.

Statistical analysis. Differences between control and experimental groups of animals were analyzed by Student's *t*-test [8]. Lines in the figures were fitted by eye.

RESULTS

Electrical potential differences and concentration ratios for passive distribution. Table 1 shows the potential differences measured between plasma and liver cells and between plasma and bile. The mean PD across the sinusoidal membrane (plasma–liver cells) was 40.3 mV with the cell negative with respect to plasma. The mean PD between plasma and bile was 3.9 mV with bile negative with respect to plasma.

Also shown in Table 1 are the concentration ratios for the passive distribution of a monovalent anion

Table 1. Measured potential differences and calculated concentration ratios for passive distribution of a monovalent anion between plasma liver cells and bile/plasma during 0.9% NaCl and sodium taurocholate infusions*

	Liver cell/plasma		Bile/plasma	
Intravenous infusion	PD (mV)	Passive concentration ratio†	PD (mV)	Passive concentration ratio
0.9% NaCl infusion	−40.3 ± 1.0	0.22	−3.9 ± 0.5	0.87
Sodium taurocholate (0.3 μmole/min)	−37.1 ± 0.5‡	0.25	−6.5 ± 0.6‡	0.78

* Means ± S.E.M. of six animals.
† The concentration ratios for passive distribution were calculated from the Nernst equation using the appropriate measured PD.
‡ Significantly different from 0.9% NaCl infusion ($P < 0.05$).

as calculated from the Nernst equation. The Nernst equation takes the following form for a monovalent anion:

$$E_m = -61.5 \log (\text{Ion})_o/(\text{Ion})_i,$$

where E_m is the measured PD and $(\text{Ion})_o$, and $(\text{Ion})_i$ are the anion activity coefficients on the outside and inside of the membrane, respectively. If the measured PD is substituted for E_m, the ratio $(\text{Ion})_o/(\text{Ion})_i$ can be calculated. This ratio is the electrochemical gradient predicted for 'passive' distribution of an anion across the membrane in the presence of the measured PD. Thus, if monovalent anions, such as PAAH or BSP, were distributed passively across the sinusoidal membrane, the observed liver cell/plasma concentration ratio would be 0.22 to 1. Deviation from this calculated ratio would suggest that active transport is partially responsible for the distribution of the anion.

Measured concentration ratios of PAAH and BSP. The observed PAAH and BSP concentration ratios in control and taurocholate-treated rats are shown in Table 2. When these values are compared to the passive concentration ratios in Table 1, it is apparent that only the bile/plasma ratio for PAAH is markedly different than predicted for passive distribution, while BSP appears to be concentrated in liver cells and bile. Sodium taurocholate infusion decreased the bile to plasma ratio of PAAH but markedly increased the ratio of BSP between bile and plasma. In control animals, bile flow was 1.94 ± 0.06 (mean ± S.E.M.) μl/min/g liver and taurocholate excretion was 21.8 ± 1.2 nmoles/min/g liver. After an i.v. infusion of sodium taurocholate (0.3 μmole/min), bile flow increased to 2.29 ± 0.11 μl/min/g liver and taurocholate excretion increased to 49.3 ± 1.8 nmoles/min/g liver.

Effect of increasing the PAAH dose on the distribution of PAAH. When the plasma concentration of unbound PAAH was increased over a 6-fold range, the biliary PAAH concentration increased (Fig. 1). Over the range of PAAH doses employed in these experiments, bile flow increased from 1.97 ± 0.1 μl/min/g liver (mean ± S.E.M.) to 2.91 + 0.05 μl/min/g liver. Furthermore, the increase in bile flow paralleled the increase in bile concentration of PAAH. Thus, the biliary excretion rate of PAAH of 19.7×10^{-3} μmoles/min/g liver, at a plasma concentration of 0.5 μmole/ml, increased to 52.4×10^{-3} μmoles/min/g liver at a PAAH plasma concentration of 2.7 μmoles/ml. The increase in the biliary PAAH concentration toward an asymptote suggests the saturation of a carrier-mediated transport system. Figure 1 also illustrates the effect of increasing the plasma PAAH concentration on the concentration of unbound PAAH in liver cell water.

Table 2. Effects of taurocholate infusion on the concentration ratios of unbound PAAH and BSP in plasma, liver cells and bile*

Intravenous infusion†	Liver cell/plasma	Bile/liver cell	Bile/plasma
		PAAH	
0.9% NaCl	0.17 ± 0.03	412 ± 99	61.8 ± 4.3
Sodium taurocholate (0.3 μmole/min)	0.13 ± 0.01	380 ± 9	47.3 ± 2.2‡
		BSP	
0.9% NaCl	5.6 ± 0.8	953 ± 105	4980 ± 385
Sodium taurocholate (0.3 μmole/min)	3.4 ± 0.6‡	2857 ± 585‡	8806 ± 1030‡

* All values represent the mean ± S.E.M. of four to six animals.
† Tracer amounts of either [^{14}C]PAAH or [^{35}]BSP were added to the treatment infusion.
‡ Significantly different from 0.9% NaCl infusion ($P < 0.05$).

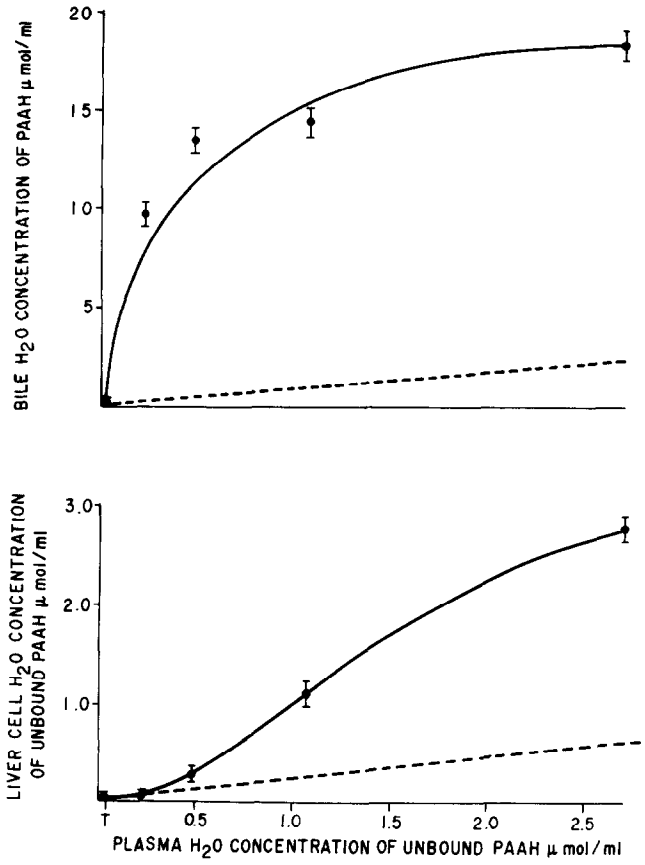

Fig.1. Effect of increasing plasma PAAH concentrations on the concentration of PAAH in bile and liver cell H_2O. In this and the following figure, the solid circles and brackets represent the means ± S.E.M. of four animals. Dotted lines illustrate the bile and liver cell concentrations (calculated from the Nernst equation) expected if PAAH were distributed passively into bile and liver cells.

A sigmoidal relation exists which is distinctly different from the hyperbola seen with the biliary PAAH concentration. The dotted lines in Fig. 1 illustrate the bile and liver cell concentrations of PAAH that should be observed if PAAH were passively distributed among plasma, liver cells and bile.

The effects of increasing the plasma PAAH concentration on the PAAH concentration ratios are shown in Fig. 2. The bile/plasma ratio decreased from 61.8 to 6.9, and the bile/liver cell ratio decreased from 412 to 6.6. The liver cell/plasma ratio increased from 0.17 to 1.06. Thus, as the transport of PAAH into bile was saturated, the bile/plasma ratio decreased toward the passive distribution ratio of 0.87. In contrast, the liver cell/plasma ratio increased away from its passive distribution ratio of 0.22.

DISCUSSION

For several years, hepatic organic anion transport, particularly of bile acids, has been regarded as being largely responsible for bile formation [9]. However, little is known about the processes which govern hepatic uptake and bilary excretion of organic anions. Much of the knowledge of organic anion excretion has come from studies of BSP. Three disadvantages accompany the use of this anion in biliary transport studies. First, BSP is conjugated to glutathione prior to bilary excretion [10, 11]. Unless the conjugates are separated and quantitated, the BSP measured in bile may bear no relationship to BSP in plasma. Indeed, it has been shown that unconjugated BSP in liver competes with the transport of conjugated BSP into bile [12]. Second, BSP is highly bound to plasma and liver cytoplasmic proteins [1, 13], making it difficult to measure accurately the unbound concentrations of BSP in plasma and liver cells. Third, BSP is associated with mixed micelles, which reduces the free concentration in bile [2]. These limitations also apply to the present study and require that the data concerning BSP transport be interpreted with caution. The use of PAAH circumvents many of these problems because it is not metabolized prior to biliary excretion in rat (see Methods), and only 36 or 48 per cent is bound to plasma or liver proteins, respectively. The association of PAAH with biliary micelles was not determined directly in these experiments. However, the fact that a taurocholate infusion, which increases the biliary output of mixed micelles, did not increase the concentration of PAAH in bile may be evidence that this organic anion was not associated appreciably with biliary micelles.

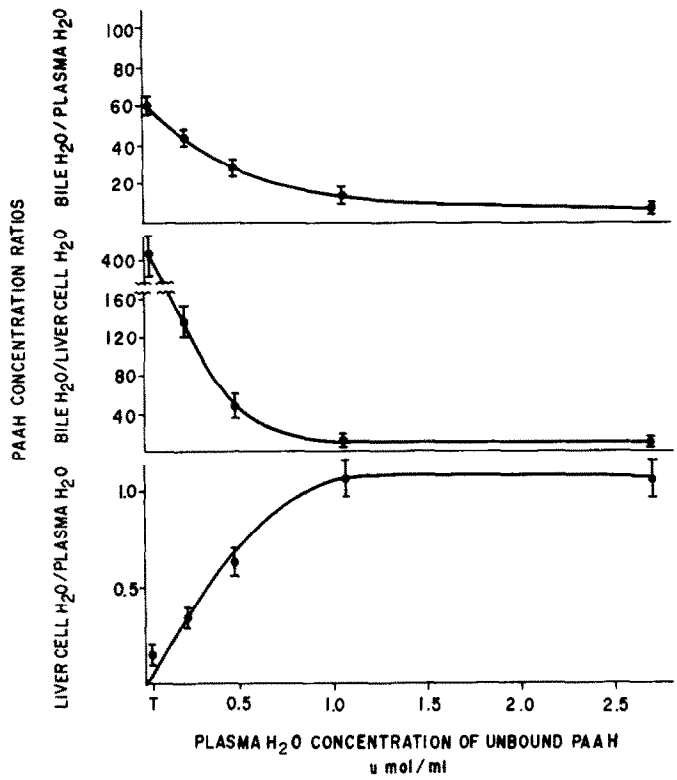

Fig. 2. Effect of increasing plasma PAAH concentration on PAAH concentration ratios between liver cell H_2O/plasma H_2O, bile H_2O/liver cell H_2O, and bile H_2O/plasma H_2O.

This study demonstrates that PAAH and BSP (with the reservations indicated above) are taken up by liver cells across the sinusoidal membrane against an electrochemical gradient. The data also suggest that these anions are transported from liver cells into bile across the canalicular membrane against an electrochemical gradient. Both membrane-transfer steps appear to be active transport processes with distinctly different characteristics. The present study also confirms a previous report that PAAH is excreted into bile by a process that can be saturated [14].

These conclusions regarding the presence of the active transport processes are based on calculations of electrochemical gradients across plasma, liver cells and bile. Measurement of the PD across a cell membrane permits the calculation of the electrochemical gradient for an anion from the Nernst equation. When a tracer dose of PAAH was administered, the bile/plasma ratio was 62. This value is markedly different from the value calculated from the Nernst equation (0.87) for the passive distribution of an anion against a negative PD of 3.9 mV. As the plasma level of PAAH was increased, the bile/plasma concentration ratio declined to a value of 6.9, indicating that the transport of PAAH across the canalicular membrane was approaching saturation. It can be predicted that complete saturation of a carrier-mediated transport system would decrease the concentration ratio to a value close to that calculated for passive distribution.

In contrast, the process of PAAH uptake across the sinusoidal membrane into liver cells is not as clearly defined. At low plasma concentrations of PAAH, the liver cell/plasma ratio is 0.17. This value is close to that predicted for passive distribution of a monovalent anion (0.22). However, as the plasma concentration of PAAH was raised to 2.7 μmoles/ml, the liver cell water PAAH concentration increased to 2.8 μmoles/ml. This concentration is four times greater than predicted on the basis of passive distribution and resulted in a liver cell/plasma concentration ratio of 1.06. When the canalicular transport system is saturated, the intracellular concentration of PAAH increases. Thus, it would appear that the canalicular transport of PAAH functions like a sink to maintain a low intracellular concentration of PAAH.

It is not possible to measure directly the PD between bile and the liver cell. Thus, the electrochemical gradient for organic anions across the canalicular membrane cannot be determined accurately. Nevertheless, the PAAH concentration ratio between common duct bile and the liver cell is 412 (see Table 2). It seems unlikely that this concentration gradient could result from the passive transfer of PAAH across the canalicular membrane. If, however, this concentration gradient was a result of passive diffusion, and it is assumed that the PAAH concentration in the common bile duct is not greatly different from that at the canaliculus, and that the distribution of PAAH across the canalicular membrane is determined solely by the PD, then the PD between bile and liver cells is estimated to be 161 mV (bile negative with respect to cell). This value

seems to be quite improbable, based on knowledge of the PD across other cell membranes and leads to the conclusion that the transfer of PAAH between liver cells and bile is not a passive process but rather an energy-requiring transport. The bile to liver cell concentration ratio of PAAH decreases to a plateau as the plasma concentration of PAAH is increased, suggesting the presence of a carrier-mediated transport system located at the canalicular membrane.

Takada *et al.* [15, 16] have studied the biliary excretion of PAAH in the rat. Their results, with low doses of PAAH, are similar to those of the present study. However, they concluded that the liver cell uptake mechanism for PAAH was unique since the liver/plasma ratio was less than 1 and speculated that binding by liver cytosol proteins might explain their results. Our data show that a liver cell/plasma ratio of less than 1 should be expected for a monovalent anion such as PAAH.

The effects of sodium taurocholate infusion on the biliary transport of PAAH and BSP are noteworthy. Taurocholate at the dose infused in these experiments decreased the bile/plasma ratio of PAAH. In contrast, taurocholate increased the bile/plasma ratio of BSP. Previous reports have shown that taurocholate increases the BSP biliary transport maximum [17, 18]. The results of this study demonstrate that taurocholate decreased the liver cell/plasma BSP ratio but markedly increased the bile/liver cell ratio. These data suggest that the major interaction between taurocholate and BSP is most likely between the liver cell and bile. It is probable that the increase in the bile to liver cell concentration ratio of BSP is partially due to an association of the organic anion with mixed micelles in bile which are increased by taurocholate infusion [2]. Since taurocholate did not increase the PAAH excretion in bile it is probable that this organic anion is not associated with mixed micelles in bile. If this is so, then PAAH would be a substance more suitable than BSP to study canalicular transport mechanisms. Specific binding sites for the organic anions bile acids [19] and BSP [20] have been located on sinusoidal and canalicular membranes of rat liver cells. It is interesting to speculate that these binding sites may be the carrier-mediated transport system proposed for PAAH.

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