

IDENTIFICATION OF AN ORGANIC ANION TRANSPORT PROTEIN IN SINUSOIDAL PLASMA MEMBRANES OF HEPATOCYTES OF THE LITTLE SKATE (R. ERINACEA) BY PHOTOAFFINITY LABELING AND KINETIC STUDIES.

G. Fricker^{§*}, G. Hugentobler[§], G. Kurz[¶], P.J. Meier[§], J.L. Boyer⁺
Universität Freiburg, F.R.G. University Hospital Zurich, Switzerland[§]; and Liver Center, Yale University⁺, New Haven, CT.

INTRODUCTION: Previous studies of hepatic organic anion transport in marine elasmobranchs indicate that despite slow rates of bile production compared to mammals, sulfobromophthalein (BSP), phenol-3,6-dibromosulfophthalein disulfonate (DBSP), taurocholic acid, bilirubin and biliverdin are all selectively removed from blood and excreted into bile in these species (Boyer et al., *Am J Physiol* 230:974-98, 1976; Grossbard et al., *J Comp Physiol B*, in press).

In rats two transport proteins for hepatic uptake of organic anions have been identified in sinusoidal membranes, a 48K protein for sodium dependent taurocholate transport (Kramer et al., *Europ J Biochem* 129:13-24, 1982; Fricker et al., *Hoppe-Seyler's Z Physiol Chem* 363:897, 1982) and a 54K protein that is multispecific for the transport of other organic anions (Fricker et al., *Hepatology* 4:762, 1984).

To gain insight into the hepatic transport system responsible for organic anion uptake in marine species, photoaffinity labeling with conjugated bile acid derivatives was performed to identify membrane components involved in these processes. Kinetic experiments were also pursued to prove whether identified bile acid binding polypeptides were directly involved in the uptake not only of bile acids but also of other organic compounds.

METHODS: Hepatocyte isolation was performed by collagenase perfusion of the isolated skate liver (Smith et al., *J Exp Zool* in press). Plasma membrane subfractions were isolated by the method of Song et al (*J. Cell. Biol.* 41:124-132, 1969) by sucrose density centrifugation. All membrane fractions were characterized by determination of specific activity of marker enzymes. A "heavy" sinusoidal membrane subfraction banded at the 1.42 M/2.07M sucrose interface in 15 membrane isolations. This subfraction was used for all photoaffinity labeling studies. Marker enzyme analysis demonstrated that this "heavy" subfraction was considerably more enriched in sinusoidal plasma membrane (Na^+, K^+ ATPase -12X) than either canalicular (Mg^{++} ATPase -4.2X, 5'-nucleotidase - 3.4X) microsomal (NADPH cytochrome c reductase - 1.3X) or mitochondrial (NADH cytochrome c reductase - 1.7X) membranes. Photoaffinity labeling of the sinusoidal membrane fraction with the sodium salt of the cholic acid photoprobe (7,7-azo-3 α -12 α -dihydroxy-5 β [3 β -³H]cholan-24-oic acid (7,7-AC) or the sodium salt of the taurocholate photoprobe (7,7-azo-3 α , 12 α -dihydroxy-5 β -[3 β ³H]cholan-24-oyl)-2-aminoethanesulfonic acid (7,7-ATC) was performed in a Rayonet RPR-100-Photoreactor (The New England Ultraviolet Co., Hamden, CT) exactly as described (Kramer et al., *Europ J Biochem* 129:13-24, 1982). Photoaffinity labeling of isolated hepatocytes was performed by

incubation of 5×10^6 cells in 2 ml of elasmobranch Ringer's solution containing 1 μ M of the respective photolabile derivative for 5 min at a wavelength of 350 nm. After washing removed unbound photolabile derivatives, the hepatocytes were disrupted and the microsomal pellet containing the plasma membrane fraction was treated with Chloroform: Isopropanol (2:1) to extract lipids prior to gel electrophoresis. The membrane fraction was then subjected to SDS-polyacrylamide gel electrophoresis. Uptake of bile acids into isolated hepatocytes was measured using a rapid centrifugation technique described previously (Smith et al., Bull MDIBL 24:82, 1984). Radioactivity was detected by liquid scintillation counting.

RESULTS: Photoaffinity labeling of membrane sinusoidal subfractions with 7,7-AC as well as with 7,7-ATC and subsequent gel electrophoresis showed an incorporation of radioactivity into one single polypeptide with an apparent molecular weight of $54,000 \pm 1,500$ ($n=15$), suggesting that only one bile-acid-binding protein was present at the sinusoidal surface of the fish hepatocyte (Fig. 1). Selective dose dependent inhibition of photoaffinity labeling by additions of taurocholate or cholate to the incubation medium indicated that the identified polypeptide interacts also with bile acids. Other organic anions, including BSP, DBSP, ICG, Probenicid and the anion exchange system blocking stilbene derivative, DIDS, decreased photoaffinity labeling indicating the existence of a multispecific anion transport system. Photoaffinity labeling studies were performed with both plasma membrane vesicles and isolated intact hepatocytes and gave similar results.

To determine whether the identified polypeptide is functionally involved in anion uptake in isolated intact hepatocyte suspensions ($n=3$), kinetic studies with cholate, taurocholate and the photolabile bile salt derivative 7,7-ATC were performed. All three compounds inhibited each others uptake competitively indicating identity of the transport system. The K_m for the uptake of cholate was determined to be ≈ 25 M, the K_m for the uptake of taurocholate and 7,7-ATC were determined to be ≈ 43 μ M and 52 μ M respectively. These data indicate a higher affinity of the transport system for the unconjugated and more hydrophobic bile salt in the physiological concentration range. Photoaffinity labeling of hepatocytes with 200 μ M nonradioactive 7,7-ATC to block the carrier, followed by washing out of unbound photolabile derivatives prior to measurement of taurocholate uptake resulted in an irreversible inhibition of bile salt uptake and a reduction in V_{max} whereas the K_m - values of the non-blocked carrier molecules remained unaffected ($n=4$). This study established that the identified polypeptide is functionally involved in bile salt uptake (Fig. 2).

Together these findings indicate that bile acids are taken up into hepatocytes of the little skate by a single transport system represented by a membrane polypeptide with the apparent molecular weight of 54,000. This is in contrast to results in rat liver, where bile acids are taken up by two different transport systems with the molecular weights of 54,000 and 48,000 (Wieland

FIGURE LEGENDS

Fig. 1. Distribution of radioactivity in polypeptides of skate hepatocytes plasma membranes (0.5 mg), photolabeled with 1 uM (5 uCi) of ^3H -7,7-ATC.

The top line represents the Coomassie-Blue stained polypeptides (absorption at 575 nm). Note the prominent labelling of a 54K protein. The 35K protein is a mitochondrial contaminant. Figure illustrates 1 of 15 experiments.

Fig. 2. Eadie-Scatchard diagram of ^3H -taurocholate uptake in isolated intact hepatocytes of the skate. The dots and solid line represent a control experiment while the open circles and dashed lines represent bile acid uptake after photoaffinity labeling of isolated hepatocytes with 200 uM 7,7-ATC. The straight lines indicate a single binding site whereas the parallel shift in the dashed line to the left indicates a reduction in the number of carrier proteins available for ^3H -taurocholate transport. Points = mean of 4 experiments.

fig 1

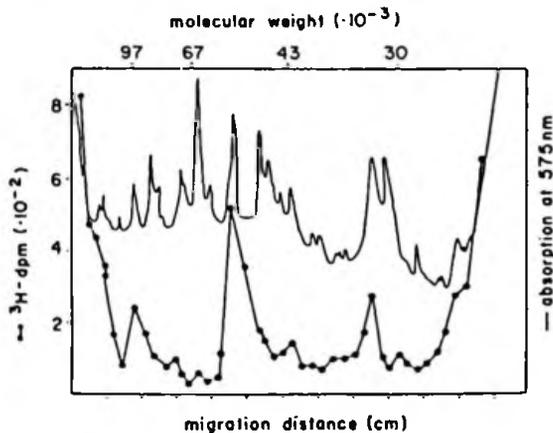
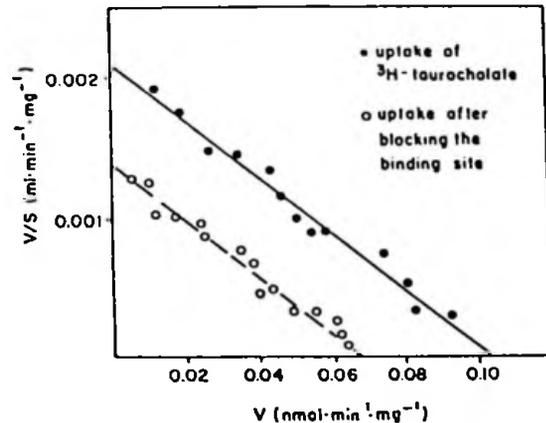


fig 2



et al., PNAS 81:5232-5238, 1984). The identified transport system in the hepatocytes of the skate is assumed to be similar to the 54,000 polypeptide in rat hepatocytes which also exhibits multispecificity for uptake of other organic anions.

Finding a 54,000 molecular weight protein for hepatic uptake of organic anions in this elasmobranch species indicates that a multispecific hepatic organic anion transport protein is expressed early in vertebrate evolution.