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Uptake of Iopanoic Acid and Its Glucuronide Conjugate by Rat Hepatocytes in Primary Culture[1] (41678)

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Abstract. Uptake of iopanoic acid (IOP) and iopanoate glucuronide (IOP-G) was studied in 3-day primary cultures of rat hepatocytes isolated by the collagenase perfusion method. ^{125}I activity of cells after incubation with ^{125}I-IOP (10–100 μM) and ^{125}I-IOP-G (10–100 μM) was used as a measure of uptake. At each concentration, uptake was linear for the first 45 sec. In the absence of albumin, the initial uptake velocity was directly proportional to the concentration or IOP or IOP-G and was nonsaturable up to 100 μM. The calculated uptake rate constants for IOP and for IOP-G were 0.059 and 0.048 nmole/(mg protein · min · μM), respectively. IOP uptake was not inhibited by sodium taurocholate nor by the contrast agents iodipamide, ipodate, and iopronic acid. The data indicate that the enhancement of IOP excretion by bile salts noted *in vivo* does not occur at the uptake step and that the hepatic uptake of both IOP and IOP-G in the absence of albumin is limited by diffusion.

Iopanoic acid (IOP) is an oral cholecystographic contrast agent which has been used for over 25 years (1). This organic anion is cleared efficiently from the blood by the liver, is conjugated with glucuronic acid to form iopanoate glucuronide (IOP-G), and is then excreted into bile. The mechanisms involved in conjugation and biliary excretion of the compound have been studied extensively (1–5). However, very little information concerning the mechanism of hepatic uptake is available. Recently, we showed in primary cultures of isolated rat hepatocytes that hepatic uptake of IOP occurs by both a passive process and a saturable process in which the saturable component was dependent upon an albumin–IOP–hepatocyte interaction (6).

Taurocholate enhances the maximum biliary excretion of IOP in both dogs (4) and rats (7), however, it is not known whether taurocholate effects the uptake, conjugation, or excretion step. The effect of taurocholate on IOP uptake by hepatocytes in primary culture was evaluated. In addition, the passive components for IOP and IOP-G uptake were compared. The results indicate that hepatic uptake of IOP was somewhat greater than the uptake of IOP-G and that IOP uptake was not influenced by taurocholate or other contrast agents.

Materials and Methods. *Chemicals.* Sodium taurocholate (Grade A) was purchased from Calbiochem–Behring Corporation, La Jolla, California. Iopanoic acid (Telepaque) was obtained from Winthrop Laboratories, New York, New York and iodipamide (Cholografin), iopronic acid (Oravue), and sodium ipodate (Oragrafin) were obtained from E. R. Squibb Company, New York, New York. Hepes buffer and collaganese were purchased from Sigma Chemical Company, St. Louis, Missouri. Eagle's basal medium with Hanks' salts (BMD) and horse serum were purchased from Grand Island Biological Company, Grand Island, New York.

The ^{125}I-IOP was purchased from New England Nuclear Corporation (specific activity of 0.7 Ci/mmole). Free iodine was removed by binding ^{125}I-IOP to XAD-7 resin, washing with 0.01 N HCl and water, eluting with methanol, and evaporating under nitrogen gas. The details are reported elsewhere (6). The radiolabeled IOP was dissolved in incubation medium containing either 0.1 or 2 mM IOP. This procedure did not cause degradation of the labeled substrate as shown by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). HPLC analyses were made using a reverse phase column (uBondapak C-18, Waters Associates, Inc.) and a mobile phase of acetonitrile/phos-

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phate buffer, 0.01 M, pH 3.0 (50/50, v/v). Butanol saturated with 2% boric acid was the solvent used for TLC analyses.

Preparation of ^{125}I-IOP-G. A cannula (PE 10) was placed in the common bile duct of an anesthetized male Sprague–Dawley rat. ^{125}I-IOP (50 μCi) was injected into a femoral vein and bile was collected for an hour. Bile containing ^{125}I-IOP-G was prepared for use in transport studies by binding to XAD-7 resin and washing with 0.01 *N* HCl and water (8). This washing procedure for ^{125}I-IOP-G is the same as was used above for preparing ^{125}I-IOP for transport studies. Studies using ^{125}I-IOP-G were performed on the same day the substrate was prepared. TLC analysis indicated the iopanoate conjugate was stable during the transport studies with no measurable amount of ^{125}I-IOP or other conjugates detected.

Primary cultures of rat hepatocytes. The technique used to prepare primary cultures of isolated rat hepatocytes was that reported by Hatoff and Hardison (9). In brief, this procedure included isolated hepatocytes by the basic collagenase perfusion technique of Berry and Friend (10) plating the cells in plastic culture dishes, allowing the cells to form a confluent layer in the bottom of the dish, and maintaining the cells in culture for 3 days. The culture medium (Hank's basic salt solution containing Hepes buffer) and the conditions for culture are the same as were reported previously (6). Cell yields ranged from 80 to 150 million cells per liver with greater than 90% viability by the trypan blue exclusion method. Approximately 500,000 cells were plated in each plastic tissue-culture well (26 × 33 mm) (Multiplate-8 well plates, Lux Scientific Corp., Newbury Park, Calif.). Cells in culture for 3 days were used for transport studies.

Transport studies. Culture dishes were removed from the incubator and washed three times with Kreb's–Ringer–bicarbonate buffer (pH 7.4, 0.15 *M*, 37°C). One milliliter of incubation solution was added to each plate and allowed to equilibrate for approximately 10 min in a shaker bath, agitated at 50 strokes/min and maintained at 37°C. Either ^{125}I-IOP or ^{125}I-IOP-G was introduced into the incubation medium to start the transport study. Transport was stopped at various time intervals by rapidly removing the incubation medium and rapidly washing the cells three times with 0.9% NaCl at 4°C (total of 15–20 ml within 10 sec) (11). Cells were removed from the plate with 1 ml of 0.2 *N* NaOH. Validation of the procedure is described elsewhere (6). ^{125}I radioactivity of the cells was determined in a gamma counter (Searle Analytic, Des Planes, Ill.). The protein content of each well was determined by the method of Lowry *et al.* (12).

To determine the initial velocity of uptake of IOP, hepatocyte cultures (approximately 0.5 mg protein per well) were incubated with 4 to 20 nCi of ^{125}I-IOP and various amounts of nonlabeled IOP to obtain final concentrations in the culture medium of from 1 to 100 μM. A similar procedure was used for ^{125}I-IOP-G transport studies.

Cells were incubated with the radioactive medium for times ranging from 0.25 to 1 min. Three or four wells were tested at each time period for each study. The accumulation of the substrate [nmole/(min · mg protein)] by the cultured hepatocytes was determined from the specific activity of the ^{125}I-IOP or ^{125}I-IOP-G in the incubation medium, the radioactivity of ^{125}I in the cells after incubation and the protein content of the cells in the test well. The initial uptake velocity was calculated from the slope of the line obtained from plotting accumulation of ^{125}I-IOP or ^{125}I-IOP-G versus time of incubation.

Statistical analysis. Linear regression method of least squares was used to calculate the values of initial uptake velocities from the respective accumulation versus time curves. Testing for significant differences between means was performed by unpaired Student's *t* test statistics with $P < 0.05$ as the minimum level of significance.

Results. Uptake of IOP by the cultured hepatocytes was nearly linear for at least 45 sec and approached a plateau after 4 min. The slope of the best-fit line obtained with the 0.25-, 0.5-, and 0.75-min points was used as the measure of the initial velocity of uptake. A plot of the initial rates of IOP uptake versus the IOP concentration in the medium is shown in Fig. 1. The initial uptake velocity increases in a linear nonsaturable fashion with increasing IOP concentrations. The uptake rate constant was 0.059 nmole/(mg protein · min · μM).

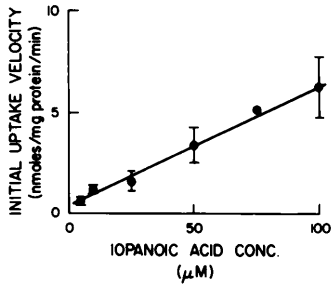

FIG. 1. Relationship between initial uptake velocity and concentration of ^{125}I-IOP in the incubation medium. Each point represents the mean ± SD of two to six experiments. Slope of line is 0.059 nmole/(mg protein · min · μM).

Extrapolation of the uptake versus time plot to zero time yields a positive value for each substrate concentration. This extrapolated value at time zero was assumed to represent nonspecific adsorption of IOP to the cultured cells. The extent of this nonspecific adsorption increased in a linear manner with respect to the substrate concentration (Fig. 2; upper panel).

Sodium taurocholate added to the incubation medium did not change the rate of IOP uptake (Table I). In additional studies, sodium taurocholate (1 mM) was added to the incubation medium containing IOP (5–100 μM) and albumin. The molar ratio of IOP and albumin was maintained at 3.4. Again, the presence of taurocholate did not alter the initial rate of IOP uptake over the range of IOP concentrations tested (Table II).

The influence of other organic anions on the initial uptake velocity of IOP was also examined to determine possible competition for uptake. Three biliary contrast agents were selected. Either iodipamide, iopronic acid, or sodium ipodate was added to the medium to establish an incubation concentration 10 times greater for the competitors than the 5 or 100 μM concentration for ^{125}I-IOP. Although the initial uptake velocities of IOP were slightly slower in the presence of the other contrast agents, they were not significantly different than the controls (Table III).

The initial uptake rate of IOP-G by the cultured hepatocytes appeared to be similar in both mechanism and magnitude as was found for IOP uptake. IOP-G uptake was nearly linear for the first minute of incubation. A plot of IOP-G uptake versus the concentration of

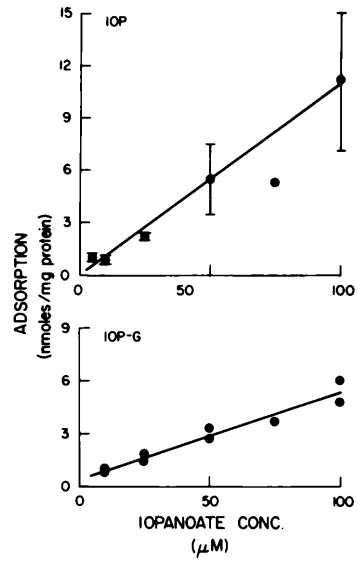

FIG. 2. Nonspecific adsorption of IOP and IOP-G to the cell surface of isolated rat hepatocytes. Upper panel: Cells incubated with ^{125}I-IOP. Each point represents the mean ± SD of two to six experiments. Slope of line is 0.111 nmole/(mg protein · μM). Lower panel: Cells incubated with ^{125}I-IOP-G. Each point represents individual experiments. Slope of line is 0.050 nmole/(mg protein · μM).

IOP-G in the incubation medium indicated that the rate of IOP-G uptake was directly proportional to the incubation concentration, that IOP-G uptake was not saturable up to a concentration of 100 μM, and that the magnitude of the uptake constant for IOP-G [0.048 nmole/(mg protein · min · μM); Fig. 3] was approximately 80% of the uptake rate constant for IOP. Nonspecific adsorption of IOP-G was also found which was approximately one-half that measured for IOP (Fig. 2; lower panel).

TABLE I. EFFECT OF TAUROCHOLATE ON THE INITIAL UPTAKE VELOCITY OF ^{125}I-IOP BY CULTURED HEPATOCYTES

IOP conc (μM)	Taurocholate conc (μM)	Initial uptake velocity nmole/(mg protein · min)
50	none	5.64 ± 0.72*
50	50	5.01 ± 1.03
100	none	8.65 ± 1.62
100	50	71.9 ± 2.58

* Mean ± SE of three separate studies.

TABLE II. EFFECT OF SODIUM TAUROCHOLATE ON INITIAL UPTAKE VELOCITY OF ^{125}I-IOP IN THE PRESENCE OF ALBUMIN (IOP:ALBUMIN = 3.4:1)

IOP incubation conc (μM)	Initial uptake velocity nmole/(mg protein · min)	
	Taurocholate (1 mM)	No taurocholate
5	0.10	0.15
10	0.34	0.55
25	0.55	0.75
50	1.28	1.30
100	3.17	2.85

Note. No statistical difference by paired t test.

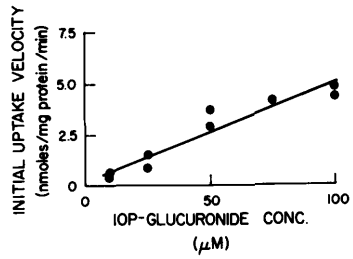

FIG. 3. Relationship between initial uptake velocity and concentration of ^{125}I-IOP-G in the incubation medium. Each point represents an individual experiment. Slope of line is 0.048 nmole/(mg protein · min · μM).

Discussion. Assessment of the individual steps of the biliary transport process for IOP indicates that a glucuronyl conjugate of IOP is formed by the action of the hepatic microsomal enzyme, glucuronyl transferase, and that it is this form of the compound (IOP-G) which is excreted into bile. In addition, evidence suggests that excretion of IOP-G into bile is a carrier-mediated process. One intriguing aspect of the hepatic disposition of IOP is the close direct link between the apparent maximum rate of biliary excretion (E_{max}) of this compound and the rate of bile salt excretion. In the dog each micromole of bile salt excreted by the liver enhances E_{max} of IOP by an additional 0.89 μmole (4). This enhancing effect of bile salt excretion on biliary transport has also been observed to a much lesser extent for other organic anions such as bilirubin (13) and sulfobromophthalein (14, 15). The mechanism involved has not been determined, although several possible mechanisms have been proposed.

Previously, mechanisms proposed to account for the effect of taurocholate on enhancing organic anion transport were aimed at one of the hepatic disposition steps following hepatic uptake and include the following: (i) an increase in micellar sink in bile to prevent back diffusion from the biliary tree (16), (ii) a recruitment of hepatocytes to provide additional transporting sites (13), (iii) an allosteric interaction with canalicular membrane to produce carrier with additional transport capacity (15), and (iv) a reduction in the toxic effect induced by the organic anion on its own biliary excretion process (17). Based on pharmacokinetic considerations, Staubus *et al.* suggested that the enhancing effect of taurocholate was likely located at a step prior to canalicular transport, either hepatic uptake or conjugation (18).

In the present study, an investigation was made as to whether an effect by taurocholate on the hepatic uptake step could account for the observed enhancing effect on IOP hepatic transport. Our findings show that the presence of 1.0 mM taurocholate in the incubation medium does not change the uptake of IOP. It is possible that taurocholate competes with IOP for binding to albumin in the medium, so that the unbound concentration of IOP would be greater resulting in a higher uptake velocity. However, taurocholate did not have any effect on the uptake of IOP in the presence or absence of albumin. Therefore, the enhancement of IOP E_{max} by taurocholate can not be attributed to an effect at the uptake step.

TABLE III. INITIAL UPTAKE VELOCITY OF ^{125}I-IOP BY CULTURED HEPATOCYTES IN THE PRESENCE OF OTHER CONTRAST AGENTS

Competitors	^{125}I-IOP initial uptake velocity nmole/(min · mg protein)	
	IOP conc = 5 μM	IOP conc = 100 μM
None	0.75 ± 0.09	7.86 ± 0.60*
Iodipamide	0.88 ± 0.13	5.71 ± 1.75
Sodium ipodate	0.56 ± 0.10	5.70 ± 0.82
Iopronic acid	0.71 ± 0.10	6.72 ± 1.71

* Mean ± SE of three separate studies.

The results of the present study are consistent with uptake of IOP in cultured hepatocytes being simple passive diffusion. IOP uptake was not saturable over the range of concentrations tested and was not affected by the presence of three other analogous organic anions in the incubation medium. Two of the compounds used as potential competitors (ipodate and iopronic acid) are oral cholecystographic contrast agents whose molecular structure is similar to IOP (19). The other is an intravenous cholangiographic contrast agent (iodipamide) which is a much more polar compound than the oral agents.

Although simple diffusion across a resistance at the cell surface appears to be the most obvious mechanism to explain these data, the anatomic identity of that rate limiting diffusional barrier is not known. The possibility that uptake of IOP is limited by diffusion across the unstirred water layer adjacent to the cultured hepatocytes, and not by diffusion across the the plasma mmbrane, can not be discounted. An estimate of the diffusion time is required to identify this barrier. The thickness of the unstirred water layer over cultured hepatocytes has not been determined. Furthermore, the technique currently used to measure uptake does not permit altering the thickness of the unstirred fluid layer in order to assess the effect of a relative change in this barrier. However, recent reports showing an enhancing effect of albumin on the hepatic uptake of rose bengal by perfused rat livers (20) and on the hepatic uptake of IOP by cultured hepatocytes (6) conclude that the effect is not due to the overcoming of an unstirred fluid barrier. For this reason, we tend to favor the plasma membrane as the likely diffusion barrier which is limiting uptake of IOP and IOP-G.

Another possible explanation for our data is that a saturable uptake process is being masked by a low-affinity, high-capacity uptake mechanism. Such a mechanism could account for the apparent linear uptake of IOP by the hepatocyte. However, support for this mechanism was not found. If this mechanism were present, the apparent maximum uptake velocity of the saturable process would be represented by the intercept value from a plot of IOP uptake velocity versus IOP concentration. Analysis of such data by our laboratory gave an intercept value of 0.07 ± 0.05 (SE) nmole/(mg protein • min) which was not different statistically from zero (6).

Uptake of IOP-G by the cultured hepatocytes appeared to be very similar to their uptake of IOP. The magnitude of nonspecific adsorption of IOP-G was only about half that for IOP indicating a difference in binding affinity between these two forms. Previous studies indicated that IOP-G has a shorter transit time between plasma and bile (8). It is possible that this occurs because IOP-G is less tightly bound to plasma proteins (7). If this is the case, then the concentration of unbound IOP-G will be higher probably resulting in a faster initial rate of entry of IOP-G by the non saturable uptake component.

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