Effects of Organic Anions on the Uptake of 1-Anilino-8-naphthalenesulfonate by Isolated Liver Cells

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Abstract □ Uptake of the fluorescent probe, 1-anilino-8-naphthalenesulfonate (I) into isolated rat liver cells was studied using both fluorescence and filtration methods. The time course of the fluorescence enhancement of I after addition to the isolated liver cells was analyzed in terms of rapid, medium, and slow phases. The slow phase (half-time ~7 min) was characteristic of viable cells. The fluorescence enhancement was proportional to the amount of I taken into the cells, as measured by the filtration method. The uptake of I followed Michaelis–Menten kinetics with an apparent K_m of 39 μM and V_{max} of 1.4 nmole/10^6 cells/min. The temperature coefficient (Q_{10}) of the uptake of I was found to be ~1.9. No pH optimum was observed, and various metabolic inhibitors did not affect the uptake of I. Among the amino acid reagents used, only 2,4-dinitrofluorobenzene decreased the uptake of I (by ~45%). The effects of various organic anions on the uptake of I were measured. The inhibition of the uptake of I by sulfobromophthalein could be analyzed in terms of competitive inhibition; the slight inhibition by sodium taurocholate could not. It is concluded that the uptake of I is a carrier-mediated facilitated process, and that the carrier is common to both I and sulfobromophthalein.

Keyphrases □ Fluorescence—measurement of 1-anilino-8-naphthalenesulfonate uptake into isolated rat liver cells, effect of organic anions, comparison with filtration method □ 1-Anilino-8-naphthalenesulfonate—determination of uptake into isolated rat liver cells using fluorescence enhancement, effect of organic anions, kinetics □ Kinetics—of 1-anilino-8-naphthalenesulfonate uptake into isolated rate liver cells, fluorescence enhancement, effect of organic anions

Sulfobromophthalein (II) and indocyanine green (III) are anionic drugs used to test liver function. The uptake mechanism responsible for the rapid and relatively selective hepatic clearance of these anions has been studied using whole animals (1–3), isolated perfused rat liver (3–5), liver slices (6), and isolated liver cells (7, 8). These studies suggested that the hepatic uptake of these anionic drugs is a carrier-mediated process.

Bile acids, endogenous compounds that are anionic at physiological pH, are also readily extracted from the plasma by the liver. Many investigators (9–11) have suggested that a carrier-mediated process might be responsible for the hepatic uptake of bile acids. If this is true, the question then arises as to whether the aforementioned anionic dyes and bile acids are transported by a common carrier. Studies on the inhibition of the uptake of one compound by another in isolated perfused rat liver (3, 5) and isolated rat liver cells (7) led to the suggestion that bile acids and other organic anions such as II and III are transported into the liver by two different systems.

The anionic fluorescent probe, 1-anilino-8-naphthalenesulfonate anion (I) has been used to study the membrane structure of several isolated membrane systems (12–14) since the large changes in its fluorescence parameters are observed in different environments (15, 16). Compound I also is an effective probe in the study of anion transport in erythrocytes (17) and Ehrlich ascites cells (18). Recently, Cheng and Levy (19) described the interaction of I with isolated rat liver cells, observing that the transport of I into liver cells was a carrier-mediated process. In the present study, we have attempted to clarify the mechanism of the uptake of I into isolated liver cells and to determine whether the transport system of I is shared by other organic anions, such as II, III, and the bile acid salts.

EXPERIMENTAL

Materials—The following analytical grade materials were used: sodium 1-anilino-8-naphthalenesulfonate[1], bromophenol[1], rose bengal[2], *p*-aminohippuric acid[2], sulfobromophthalein[3], indocyanine green[3], phenolsulfonphthalein[3], sodium taurocholate[4], sodium oleate[1], potassium cyanide[2], *n*-ethylmaleimide[2], 2,4-dinitrofluorobenzene[2], rotenone[4], carbonylcyanide *m*-chlorophenylhydrazone[4], *p*-hydroxymercuribenzoate[4], trypan blue[2], and collagenase (type I)[4].

Isolation of Liver Cells—Liver cells were isolated using a modification of the procedure of Baur *et al.* (20). Male Wistar rats (260–330 g), given free access to food and water, were used. During preparation, the liver was perfused with a calcium-free perfusion buffer at 37° *via* the vena portae (35 ml/min for 15 min). After preparation of the liver, perfusion was continued with a recirculating medium containing 4 mM calcium and 0.05% collagenase. After 15–20 min, the vena portae began to leak; the perfusion was continued *via* the vena cava for another 10–15 min. After perfusion for ~30 min, the liver disintegrated. Subsequently, the tissue was transferred to a round-bottomed flask and enzymatic treatment was continued for another 10 min with slow stirring. After washing, the cells were stored as a suspension (5×10^6 cells/ml) at 0° in a standard buffer saturated with 95% O_2 and 5% CO_2[5]. The composition of the perfusion buffer was: 121 mM NaCl, 6 mM KCl, 0.6 mM $MgSO_4$, 12 mM $NaHCO_3$, 0.74 mM KH_2PO_4, and 5 mM glucose (pH 7.2–7.1). The composition of the standard buffer was: 131 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$, 3 mM Na_2HPO_4, and 10 mM Tris/HCl (pH 7.4).

Cell viability for each experiment was determined by the trypan blue exclusion test; the value obtained usually ranged from 93–98%. Damaged liver cells (<9% viability) were prepared by treatment in an automatic mixer for >5 min.

Uptake Studies—*Filtration Method*—Aliquots of the stock cell suspensions were diluted in 5 ml of the standard buffer to a final concentration of 5×10^5 cells/min. After preincubation for 3 min at 25°, small aliquots (3–35 μl) of a 5 mM solution of I were added, and the mixtures were incubated for various times. The uptake process was terminated by vacuum filtration of 1 ml of the suspension using a glass-fiber membrane[6]. After washing the cells on the filter three times with 3 ml of the ice-cold standard buffer, the content of I in the cells was analyzed spectrofluorometrically[7]. The cells were extracted with 4 ml of solvent (methanol-1 N NaOH, 8:1) for 1 hr. After centrifugation[8] at 3000 rpm for 15 min, the supernatant solution was analyzed spectrofluorometrically at 500 nm (excitation at 390 nm). The recovery of I from the liver cells was nearly 100%.

Fluorescence Method—The fluorescence measurements[7] were performed at room temperature (20–22°), unless otherwise stated, with 10-nm slit widths for both excitation and emission channels and a 430-nm

[1] Tokyo Chemical Industries, Co., Tokyo, Japan.
[2] Wako Pure Chemical Co., Tokyo, Japan.
[3] Daiichi Chemical Co., Tokyo, Japan.
[4] Sigma Chemical Co., St. Louis, Mo.
[5] Carbogen, Takachiho Shoji Co., Tokyo, Japan.
[6] GF/F membrane, Whatman.
[7] Hitachi MPF-4 fluorescence spectrometer.
[8] Kubota centrifuge.

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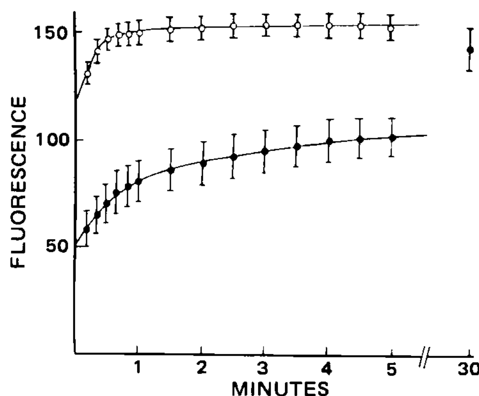

Figure 1—*Time course of the fluorescence enhancement of I (38 μM) added to isolated liver cells (5 × 10^5 cells/ml) in the standard buffer (pH 7.4) at room temperature. Excitation is at 400 nm; emission is at 480 nm; fluorescence is in arbitrary units. Each point is the mean of five experiments; bars represent the standard error. Key: (●) intact cells (93–98% cell viability); (○) damaged cells (3–9% cell viability).*

cutoff filter. The excitation and emission wavelengths for I were 400 and 480 nm, respectively. If necessary, fluorescence intensities were corrected for the inner filter effect according to the method of Chignell (21).

Small aliquots of a solution of I were added to the cell suspension (5 × 10^5 cells/ml) in the standard buffer. The solution was preincubated for 5 min and quickly mixed with a small stirring rod, and the fluorescence intensity was recorded at a chart speed of 12 cm/min. The mixing procedure could be performed in <4–6 sec. In some experiments, after the time course of uptake had been recorded for 30 sec after the addition of I, the zero point was lowered by adjusting the zero-suppression knob, and the sensitivity was increased so that the change of fluorescence could be monitored more closely.

In the experiments on inhibition by other organic anions, inhibitors were added 10 sec before the addition of I. In the experiments on inhibition by metabolic inhibitors and amino reagents, the reagents were added at the beginning of the 5-min preincubation.

Kinetic Analysis of the Data Obtained by the Fluorescence Method—For reasons discussed subsequently, the initial uptake rate of I was obtained from the initial slope of the slow component of fluorescence enhancement (40–100 sec). If the change of fluorescence (ΔF_{cell}) is assumed to be proportional to the change of I content within the cells (ΔA_{cell}), then:

$$\Delta F_{cell} = q_{cell} \cdot \Delta A_{cell} \qquad \text{(Eq. 1)}$$

where q_{cell} is the proportionality coefficient, which corresponds to the quantum yield of I bound to the intracellular components. [The validity of this assumption will be demonstrated later (*Results*).] The initial uptake rate in a carrier-mediated transport system can be expressed by the Michaelis–Menten-type equation:

$$\overline{\Delta A}_{cell} = \frac{V_{max} \cdot (A)}{K_m + (A)} \qquad \text{(Eq. 2)}$$

where $\overline{\Delta A}_{cell}$ is the initial uptake rate (40–100 sec), K_m is the apparent Michaelis constant, V_{max} is the maximum rate of uptake, and (A) is the concentration of I in the medium. Substituting Eq. 2 into Eq. 1 gives:

$$\overline{\Delta F}_{cell} = \frac{\overline{\Delta F}^{max}_{cell} \cdot (A)}{K_m + (A)} \qquad \text{(Eq. 3)}$$

where:

$$\overline{\Delta F}^{max}_{cell} = q_{cell} \cdot V_{max} \qquad \text{(Eq. 4)}$$

and $\overline{\Delta F}_{cell}$ is the initial rate of fluorescence enhancement (40–100 sec).

The binding of I to the plasma membrane of liver cells was determined by extrapolating the uptake curves to time zero, as described previously (7, 9, 11, 22). The kinetic analysis was carried out in a similar way, using:

$$F_{zero} = \frac{F^{max}_{zero} \cdot (A)}{K_{pm} + (A)} \qquad \text{(Eq. 5)}$$

and

$$F^{max}_{zero} = n \cdot q_{membrane}(P_{cell}) \qquad \text{(Eq. 6)}$$

Here, F_{zero} is the fluorescence intensity obtained by extrapolating the uptake curves to time zero, $q_{membrane}$ is the proportionality coefficient (which corresponds to the quantum yield of I bound to the plasma membrane), K_{pm} is the dissociation constant, (P_{cell}) is the concentration of cells, (A) is the concentration of I in the medium, and n is the number of binding sites for I on a cell. Kinetic parameters were calculated with a program (23) for least-squares fitting, using a computer[9].

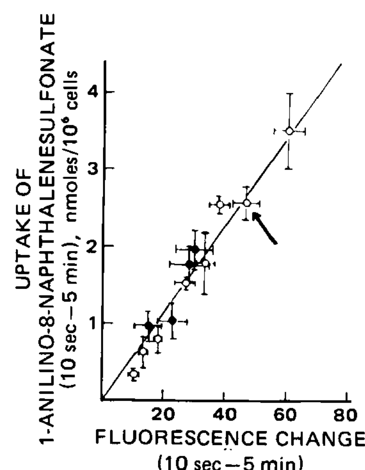

Figure 2—*Comparison of measurements of I uptake by the fluorescence and filtration methods using the same cell preparation. Small aliquots (3–35 μl) of I were added to the cell suspension (5 × 10^5 cells/ml), and the fluorescence change from 10 sec to 5 min was measured (abscissa), as described in Fig. 1. The amount of I taken into the cells during the same period was measured by the filtration method (ordinate). Each point is the mean of 4–5 experiments; bars represent the standard error. Key: (○) in the absence of II (concentration of I, 4.7–54 μM); (●) in the presence of II, added 10 sec before the addition of I (concentration of I, 38 μM; concentration of II, 20–60 μM). The arrow shows the result at a I concentration of 38 μM in the absence of II (control).*

RESULTS

Time Course of I Uptake into Intact and Damaged Cells—When 38 *μM* I was added to hepatocytes (5 × 10^5 cells/ml), a marked enhancement of the fluorescence of I was observed. The fluorescence of I in buffer alone was negligible when compared with that obtained in the presence of hepatocytes. Figure 1 shows the time course of fluorescence enhancement of I both in intact and damaged cells. The time course in damaged cells (viability <9%) showed a rapid phase (half-time less than the mixing time) and a time-dependent (medium) phase which had almost reached a plateau within 40 sec. In the case of intact cells, a new time-dependent kinetic phase (slow phase) appeared, and the fluorescence intensity continued to increase up to 30 min. This slow phase, which is characteristic of intact cells, is thought to reflect the uptake of I and subsequent binding to intracellular components, while the rapid phase may correspond to the binding of I to plasma membrane. On this basis, the initial uptake rate was calculated from the initial slope of the slow phase of fluorescence enhancement (40–100 sec), as shown in Fig. 1.

Comparison of the Data Obtained by the Fluorescence and the Filtration Methods—To analyze the uptake of I by the fluorescence method, the validity of Eq. 1 must be demonstrated. Therefore, the changes of the fluorescence of I (10 sec–5 min) were compared with the amounts of I transported into cells during that period, as directly measured by the filtration method (Fig. 2). Figure 2 shows that the change of fluorescence (ΔF_{cell}) is proportional to that of I content within cells (ΔA_{cell}). The q_{cell} value in Eq. 1 was calculated to be 18.7/nmole of I from the data in Fig. 2.

The cell-medium ratio (C/M) of I after the establishment of equilibrium between the cells and the medium (~30 min after the addition of I) was calculated both from the data in Fig. 1 and from the q_{cell} value. In calculating the intracellular concentration of I, an intracellular volume of 4.2 μl/10^6 cells was used (24). C/M values of 45 ± 4 and 36 ± 2 were calculated for initial I concentrations of 4.7 *μM* and 39 *μM*, respectively.

Kinetic Analysis of the Initial Uptake Rate and Binding to Plasma Membrane at Various Concentrations of I—The initial rates (40–100 sec) of I uptake at concentrations ranging from 4.7 to 78.5 *μM*

[9] Hitachi 8700/8800 computer.

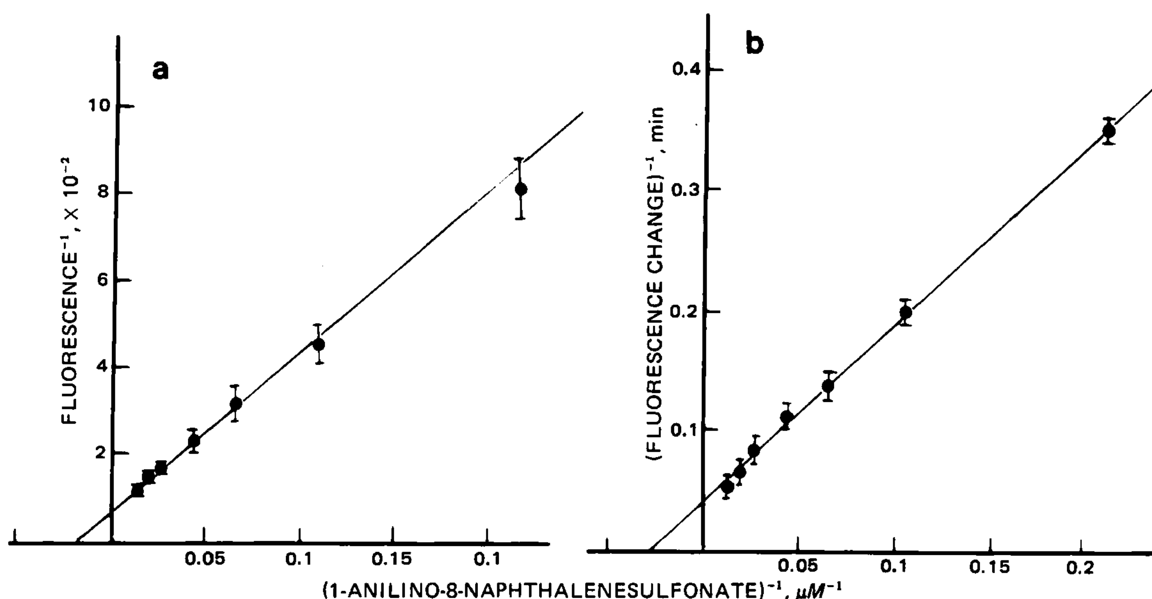

Figure 3—*(a) Double-reciprocal plot of I binding to the plasma membrane, determined by extrapolating the curve of fluorescence enhancement to time zero. The line represents the regression line calculated using Eq. 5. (b) Lineweaver–Burk plot of I uptake with the initial rate calculated from the initial slope (40–100 sec) of the slow phase (Fig. 1). The line represents the regression line calculated using Eq. 3. The fluorescence was measured at room temperature in the standard buffer with a cell concentration of 5 × 10^5 cells/ml. Each point is the mean of 5–9 experiments; bars represent the standard error.*

were measured by the fluorescence method. Figure 3b shows a Lineweaver–Burk plot of the uptake rates based on Eq. 3. An apparent K_m of 38.8 ± 5.2 μM and $\overline{\Delta F_{cell}^{max}}$ of 26 ± 2.8 min^{-1} were obtained. A V_{max} of 1.39 ± 0.15 nmole/10^6 cells/min was calculated from the $\overline{\Delta F_{cell}^{max}}$ and the q_{cell} values (18.7/nmole of I).

The binding of I to the plasma membrane of liver cells was determined by extrapolating the uptake curves to time zero. The data thus obtained were analyzed by means of a double-reciprocal plot based on Eq. 5, as shown in Fig. 3a. A dissociation constant (K_{pm}) of 56.7 ± 5.5 μM was obtained.

Effects of Metabolic Inhibitors and Amino Acid Reagents—To determine whether the uptake of I is dependent on metabolic energy, 19 μM I was added to cell suspensions preincubated for 5 min with one of four metabolic inhibitors, and the initial enhancement of fluorescence (40–100 sec) was measured. The inhibitors were potassium cyanide (1 mM), which blocks electron transport at the final step; rotenone (10 μM), which blocks electron transport near the initial step; ouabain[4] (1 mM), which inhibits Na^+-K^+ ATPase and the oxidative phosphorylation uncoupler carbonylcyanide *m*-chlorophenylhydrazone (10 μM). None of the inhibitors had a detectable effect on the initial uptake of I. Consequently, the concentrative uptake of I (C/M = 36–45) described previously is not due to the active transport of I, but to its binding to intracellular components.

The effects of amino acid reagents, which have been successfully used in membrane and cell research to inhibit carrier-mediated processes, were measured by the fluorescence method. *p*-Hydroxymercuribenzoate (100 μM) and *n*-ethylmaleimide (100 μM), which bind covalently to free sulfhydryl groups, did not significantly reduce the initial uptake of I (concentration of I, 19 μM); 2,4-dinitrofluorobenzene (100 μM), which binds covalently to free amino groups, decreased the initial uptake of I by 47 ± 7% (mean ± *SE* for three separate experiments). To confirm its effect, 2,4-dinitrofluorobenzene was also studied by the filtration method, which gave similar results, *i.e.*, a reduction ratio of 43% (n = 2).

Effect of Temperature and pH—In viable cells, the initial enhancement of fluorescence (40–100 sec) at 35° was higher than that at 25° (Fig. 4). The temperature coefficient ($\Delta F_{35°}/\Delta F_{25°}$) was 1.43 ± 0.12 ($n$ = 3). The dependence of the initial uptake of I on temperature was also measured by the filtration method, and a temperature coefficient (Q_{10}) of 1.9 ± 0.3 (n = 3) was obtained. The methods seem to give slightly different coefficients. The most likely explanation for this discrepancy is that the q_{cell} value in Eq. 1 changes with temperature. In damaged cells, the fluorescence intensity obtained after establishment of equilibrium at 25° was higher than that at 35° ($F_{35°}/F_{25°}$ = 0.83 ± 0.05). This result

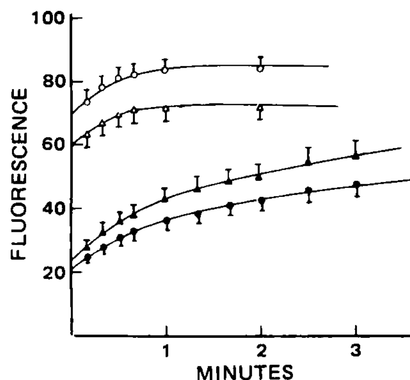

Figure 4—*Effect of temperature on the time course of fluorescence enhancement when I (19 μM) was added to a cell suspension (5 × 10^5 cells/ml) in the standard buffer. Each point is the mean of three experiments; bars represent the standard error. Key: (●) intact cells, 25°; (▲) intact cells, 35°; (○) damaged cells, 25°; (△) damaged cells, 35°.*

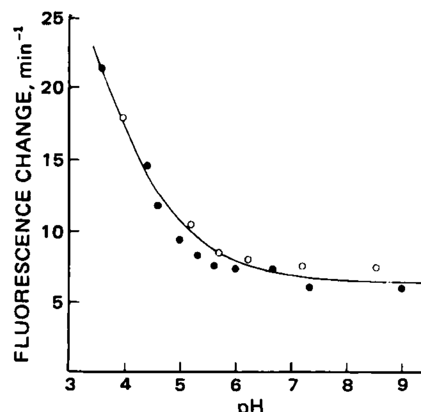

Figure 5—*Effect of pH on I uptake, using the standard buffer brought to various pH values. An aliquot of stock cell suspension (1 × 10^8 cells/ml) was diluted with the medium at each pH to yield a final concentration of 5 × 10^5 cells/ml prior to the addition of I (19 μM). The initial rates of uptake were measured by the fluorescence method as described in Fig. 3b. After the fluorescence measurement, the pH of each suspension was remeasured. Open and closed circles represent results using different cell preparations.*

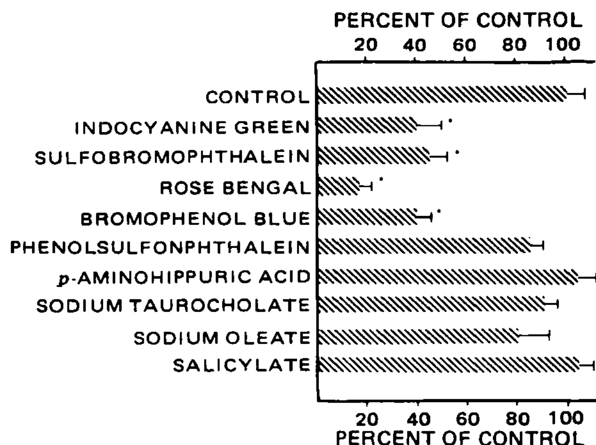

Figure 6—*Inhibitory effects of various organic anions at concentrations of 20 μM. Inhibitors were added to the cell suspension in the standard buffer (5 × 10^5 cells/ml) 10 sec before the addition of I (19 μM). Initial rates of uptake were measured by the fluorescence method as described in Fig. 3b. Fluorescence intensities were corrected as necessary for the inner filter effect. Each point is the mean for 3–4 experiments; bars represent the standard error; asterisks indicate statistical significance at the $p < 0.05$ level.*

in damaged cells might reflect a change of q_{cell} with temperature. On this basis, the Q_{10} obtained by the fluorescence method was recalculated as 1.75 (1.43/0.83), which is in good agreement with the value obtained by the filtration method.

Figure 5 shows the pH dependence of the initial uptake rate of I (concentration, 19 *μM*) measured by the fluorescence method. Between pH 6 and 9, the fluorescence was considerably increased.

Effects of Organic Anions—The initial uptake rate of 19 *μM* I was determined by the fluorescence method in the presence of various organic anions, each at 20 *μM* concentration (Fig. 6). Four organic anions (II, III, bromophenol blue, and rose bengal), which are thought to act as substrates for an organic anion transport system in the liver, inhibited the uptake of I by >50%; sodium taurocholate, which is thought to be transported into the liver by a different system (3, 5), did not show significant inhibition. *p*-Aminohippuric acid and phenolsulfonphthalein, which are known to act as substrates for an organic anion transport system in the kidney, did not have a significant effect.

Compound II and sodium taurocholate were selected as typical anions transported into the liver by different systems, and kinetic investigations of the inhibition of I uptake using these compounds were carried out. First, the effect of the addition of II on the q_{cell} value in Eq. 1 was examined (Fig. 2). Although both the fluorescence and filtration methods showed inhibition of I uptake by II, the q_{cell} value was not significantly altered by II. This finding indicates that it is possible to analyze the inhibition of I uptake kinetically by the fluorescence method.

In Fig. 7, the reciprocal of the initial rate of fluorescence enhancement

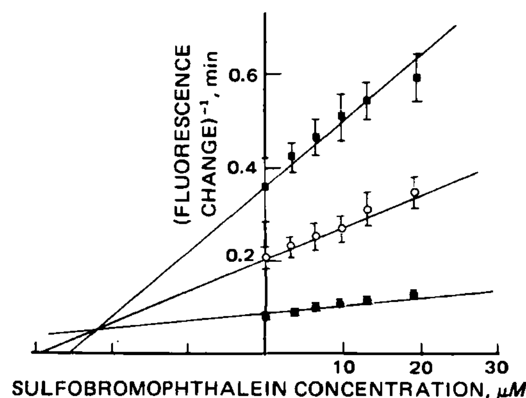

Figure 7—*Dixon plot of I uptake in the presence of II, measured by the fluorescence method, as described in Fig. 3b. Compound II was added 10 sec before the addition of I to the cell suspension (5 × 10^5 cells/ml). Each point is the mean of three experiments; bars represent the standard error. The lines represent the regression lines calculated using Eq. 7. Key: concentration of I (■) 4.7 μM; (○) 9.4 μM; (●) 38 μM.*

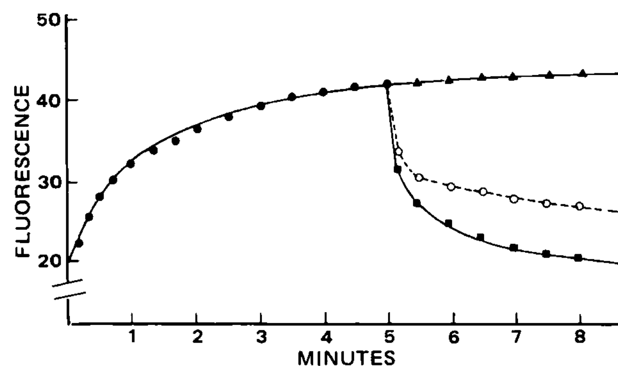

Figure 8—*Countertransport study using a small aliquot of buffer (▲), 20 μM II (○), or 20 μM III (■) added to the cell suspensions (5 × 10^5 cells/ml) after incubation with 19 μM I for 5 min. Each point is the mean of two experiments. The data points (●) before 5 min are means taken from the uptake experiments.*

of I (40–100 sec) is plotted *versus* the concentration of II. This Dixon plot shows that the uptake of I is inhibited competitively by II. The kinetic parameters $K_m = 32.7 \pm 6.5\ \mu M$, $K_i = 22.2 \pm 2.7\ \mu M$, and $\overline{\Delta F_{cell}^{max}} = 22.2 \pm 4.3\ \text{min}^{-1}$ were obtained by fitting the data to an equation that corresponds to competitive inhibition:

$$\overline{\Delta F_{cell}} = \frac{\overline{\Delta F_{cell}^{max}} \cdot (A)}{K_m(1 + i/K_i) + (A)} \qquad \text{(Eq. 7)}$$

where i is the concentration of inhibitor (II), K_i is the inhibition constant, and the other symbols are the same as those in Eqs. 3 and 4. The kinetic parameters thus obtained ($K_m = 32.7\ \mu M$ and $\overline{\Delta F_{cell}^{max}} = 22.2\ \text{min}^{-1}$) are in good agreement with those ($K_m = 38.8\ \mu M$ and $\overline{\Delta F_{cell}^{max}} = 26.0\ \text{min}^{-1}$) obtained from an independent uptake study of I (Fig. 3a).

On the other hand, inhibition by sodium taurocholate could not be detected in Fig. 5, so the inhibition of I uptake (19 *μM*) at a higher concentration of sodium taurocholate was measured. Even 170 *μM* sodium taurocholate inhibited the uptake of I by only 20% (mean of two separate experiments). If it is assumed that the uptake of I is competitively inhibited by sodium taurocholate and that the K_i value is 19 *μM*, 20 and 170 *μM* sodium taurocholate should inhibit the uptake of I by 41 and 86%, respectively[10]. A Michaelis constant of 19 *μM* was obtained by Schwarz *et al.* (9) in a study of taurocholate uptake into isolated liver cells. Marked discrepancies between the observed and calculated values suggest that the uptake system of sodium taurocholate is different from that of I.

Countertransport—Compound II was added to the cell suspension after addition of I (Fig. 8). The addition of II induced a rapid decrease of fluorescence which was complete within 10 sec, followed by a slower decrease. The rapid decrease suggests the inhibition of the plasma membrane binding of I by II. The slower decrease of fluorescence may represent the efflux of I previously taken into the cells, reflecting countertransport (1, 25, 26). A similar effect was also seen when III was added instead of II.

DISCUSSION

Many studies on the hepatic uptake of various compounds utilize isolated liver cells, and the advantage of their use has been discussed (7, 8, 27). In most of these studies, cells were separated from the incubation medium by centrifugation or filtration. The present study, using a fluorescence probe (I) as a substrate, has the advantage that the uptake of I can be determined simply by measuring the fluorescence change, without separation. This method has already been adopted in studies of anion transport in erythrocytes (17), Ehrlich ascites cells (18), and isolated liver cells (19).

The time course of fluorescence enhancement of I (Fig. 1) was analyzed by means of a semilogarithmic plot of log [fluorescence change (total

[10] Equation 7 yields the following equation:
Ratio of fluorescence change (R)

$$= \frac{\overline{\Delta F_{cell}} \text{ in the presence of sodium taurocholate}}{\overline{\Delta F_{cell}} \text{ in the absence of sodium taurocholate}} = \frac{K_m + (A)}{K_m(1 + [\text{sodium taurocholate}]/K_i) + (A)}$$

where $K_m = 38.8\ \mu M$, $(A) = 19\ \mu M$, and $K_i = 19\ \mu M$.

minus partial)] against time. This method is known to give good fits to kinetic data obtained in experiments with erythrocyte ghosts (28) or submitochondrial particles (29). The plot in the case of intact cells could be analyzed in terms of a three-exponential equation (not shown): *i.e.*, the fluorescence enhancement consisted of a rapid phase (half-time less than the mixing time), a medium phase (half-time of ~15 sec), and a slow phase (half-time of ~7 min). The rapid, medium, and slow phases comprised ~35, 20, and 45% of the overall fluorescence enhancement, respectively. Damaging the cells by vigorous shaking (viability <9%) hardly affected the half-time of the medium phase, but abolished the slow phase. A half-time (~15 sec) corresponding to the medium phase in the present study was also found in studies of the interaction of I with erythrocyte ghosts (28) and plasma membranes of hepatoma tissue culture cells (19), both of which lack intracellular components. Our findings and those of other workers suggest that the rapid phase corresponds to the binding to superficial sites of the plasma membrane, the medium phase to the binding to deep sites within the plasma membrane, and the slow phase to the uptake and subsequent binding to intracellular components. We assumed that the permeability barrier to I would be destroyed in damaged cells, and used the slow phase for measurement of the initial uptake of I. Cheng and Levy (19) used the period from 30 to 120 sec in uptake studies of I into isolated liver cells. However, in the present studies, we used 40–100 sec for measurement of the initial rate of uptake as the plot of fluorescence enhancement against time showed curvature after 100 sec (Fig. 1). Furthermore, we used 40 sec as the starting time of the slow phase to avoid the contribution of the medium phase.

Before the fluorescence method can be accepted for the measurement of I uptake, it must be demonstrated that the fluorescence change is proportional to the amount of I taken into the cells, *i.e.*, the q_{cell} value in Eq. 1 should be constant regardless of the I concentration within the cell. The results shown in Fig. 2, which demonstrate the validity of Eq. 2, confirm this method. The q_{cell} value (18.7/nmole of I) was obtained from the data between 10 sec and 5 min (Fig. 2); however, strictly speaking, the q_{cell} value should be obtained using only the slow phase, which is thought to reflect the true uptake phase. On the other hand, we also compared the changes of I fluorescence (0–10 sec) at several concentrations of I in a similar way. This period is thought to reflect the binding to the plasma membrane. A $q_{membrane}$ of 23/nmole of I was obtained in this experiment (unpublished data). This $q_{membrane}$ value, which corresponds to the quantum yield of I bound to the plasma membrane of liver cells, is not very different from the q_{cell} value (18.7/nmole of I). This result suggests that the quantum yields of I are essentially the same whether I is bound to the plasma membrane or to the intracellular components. Therefore, the q_{cell} value obtained from the data between 10 sec and 5 min may be considered to represent the true q_{cell} value. Moreover, the q_{cell} value was not altered by II (Fig. 2), and this result made it possible to investigate the inhibition of I uptake by II using the fluorescence method. To our knowledge, this is the first experiment demonstrating the validity of the fluorescence method.

Several specific criteria must be met to demonstrate the presence of carrier-mediated transport across biological membranes: the transport system must be saturable, structurally similar compounds should inhibit the transport process, and the system should show countertransport. These criteria are common to both active-transport and facilitated-diffusion processes. To differentiate between these two types of transport, other criteria are required.

By definition, active transport differs from facilitated diffusion in two ways: (*a*) transport of a substrate can occur against a chemical potential and (*b*) an expenditure of metabolic energy is required and the process generally is associated with a temperature coefficient (Q_{10}) of ≥ 3 (25). Each of these criteria was investigated in detail for the I uptake into isolated liver cells. The saturability (Fig. 3b) and the competitive inhibition by II (Fig. 7) suggest that a carrier-mediated transport system may be responsible for the uptake of I; saturability of I uptake was also suggested by Cheng and Levy (19). A countertransport study was undertaken to confirm the presence of carrier-mediated transport. The results obtained with II and III suggest countertransport (Fig. 8). However, these results should be interpreted with caution, since the phenomena shown in Fig. 8 might occur if II and III simply displace I from the binding sites of I on the intracellular components. This displacement would increase the unbound concentration of I within the cells and, thus, accelerate the efflux of I to the medium. Therefore, the fluorescence change caused by II and III shown in Fig. 8 is necessary but not sufficient to demonstrate true countertransport.

In addition to these findings, the inhibition of I uptake by 2,4-dinitrofluorobenzene, which combines with free amino groups on the carrier molecule, strongly suggests the countribution of a carrier-mediated process to uptake of I. It has been shown that sulfhydryl reagents can inhibit the uptake of cortisol (30) and procainamide ethobromide (31) into isolated liver cells. In the present study, *p*-hydroxymercuribenzoate and *n*-ethylmaleimide did not have a significant inhibitory effect. This may indicate the absence of a functional sulfhydryl group at the active site of the carrier protein for I. However, the uptake of I was unaffected by various metabolic inhibitors, suggesting that active transport is not involved. Independence of metabolic energy has also been demonstrated by Schwenk *et al.* (7) in the uptake of II into isolated liver cells. Furthermore, the relatively small dependence of I uptake on temperature ($Q_{10} = 1.9$) also suggests a facilitated-diffusion process. A similar temperature dependency (activation energy of 11 kcal/mole, Q_{10} of ~2) was reported in the uptake of II into isolated liver cells (7). Based on these findings, which suggest the facilitated diffusion of I, the accumulation of I in the cells (C/M of 36–45) found in the present study could be regarded as due to the binding of I to the intracellular components. In fact, I is bound to the cytoplasmic binding proteins (X, Y, and Z fraction) in the liver, especially to the Z-protein (32).

With regard to pH, the uptake of I (Fig. 5) is similar to that of II (7). Both have no pH optimum, though the uptake was considerably increased below pH 6. As described above, the uptake of I resembles that of II in many respects, namely the effect of metabolic inhibitors, the temperature dependence, and the pH profile. On the other hand, the uptake of sodium taurocholate was inhibited by metabolic inhibitors (carbonylcyanide *m*-chlorophenylhydrazone, ouabain, and antimycin A), the activation energy amounted to 29 kcal/mole which corresponds to a Q_{10} of ~5, and the uptake showed a pH optimum between pH 6.5 and pH 8.0 (9).

The similarity of the uptake of I to that of II raised the possibility that both compounds share a common carrier. To test this possibility, the effect of II on the uptake of I was studied kinetically (Fig. 7). A Dixon plot showed that uptake of I is inhibited competitively by II, indicating a common carrier for the uptake of both compounds. The K_i value (22 μM) of II obtained in the present study is comparable with the K_m value (7 μM) for II obtained by Schwenk *et al.* (7). On the other hand, the slight inhibition of I uptake by a high concentration of sodium taurocholate (170 μM) could not be explained by competitive inhibition. These findings indicate that I and II are transported by the same carrier, and that this carrier is different from that for sodium taurocholate. This result supports the hypothesis of Paumgartner and Reichen (3, 5) that there are at least two transport systems for organic anions: one for bile acids and the other for organic anions such as II and III.

Recently, a high-affinity binding protein for II (thought to be the carrier protein) has been isolated from the plasma membrane of the rat liver (33–35). During this isolation, the binding activity at each step was evaluated by gel-filtration using a small column (33) or equilibrium dialysis (34). The present result that I and II share a common carrier raised the possibility that fluorescence measurements of I could provide a simple and novel method for detecting binding protein for organic anions such as II and III during the isolation procedures.

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Thiazides VIII: Dissolution Survey of Marketed Hydrochlorothiazide Tablets

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Received April 12, 1982, from the **Department of Pharmaceutics, University of Maryland School of Pharmacy, Baltimore, MD 21201* and the ‡*Biopharmaceutics Laboratory Branch, Washington, D.C. 20204.* Accepted for publication, July 20, 1982. §Present address: Wyeth Laboratories, Philadelphia, PA 19101

Abstract □ The dissolution profiles of 50-mg hydrochlorothiazide tablets representing all approved manufacturers (at the time of the study) were determined in two vehicles [purified water and dilute (1:100) hydrochloric acid] by three methods (rotating basket at 150 rpm; spin filter at 300 rpm; paddle method at 50 rpm). The paddle method was preferred on the basis of overall ease of operation, reproducibility, and discrimination. The paddle data were validated in both vehicles on the same lots of tablets by a second laboratory. A standard of not <80% dissolution in 60 min by the paddle method in water is proposed for hydrochlorothiazide tablets.

Keyphrases □ Hydrochlorothiazide tablets—dissolution studies, paddle method, basket method, spin filter method □ Dissolution—paddle method, basket method, spin-filter method, hydrochlorothiazide tablets □ Paddle method—dissolution of hydrochlorothiazide tablets □ Basket method—dissolution studies of hydrochlorothiazide tablets □ Spin-filter method—dissolution studies of hydrochlorothiazide tablets

Hydrochlorothiazide is a member of the benzothiadiazine class of orally effective diuretics widely used in the treatment of hypertension, congestive heart failure, and other edematous conditions. As a class, these compounds generally are poorly wetted and have limited solubility. Thus, it is not surprising that they have been identified in the Federal Register (1) as a class of drugs with a potential for bioavailability/bioequivalency problems and for which dissolution standards should be developed. For such standards to be meaningful and reflect bioavailability performance, the dissolution system must be capable of generating data that consistently correlate with *in vivo* performance. However, the dissolution of a drug from its dosage form is dependent on many factors, which include not only the physicochemical properties of the drug, but also how the dosage form is formulated and processed. Thus, even in the absence of a correlation between *in vivo* and *in vitro* data, dissolution data provide a desirable aid in controlling formulation and manufacturing variables and should be a reliable indicator of uniformity of manufacture. The objectives of this study were to (*a*) survey the dissolution performance of marketed hydrochlorothiazide products by various methods, (*b*) select an appropriate dissolution method, and (*c*) develop acceptable dissolution standards based on the performance of the marketed products. The results of this study should form a basis for the consideration of other members of the benzothiadiazine class.

EXPERIMENTAL

Materials—Commercial 50-mg hydrochlorothiazide tablets[1] (representing all approved manufacturers at the time of the study) were used.

Reagents and Chemicals—Distilled water was used throughout, and all other chemicals or reagents were either official grade or reagent grade. Hydrochlorothiazide USP was the reference standard.

[1] Product 1, Abbott Laboratories Lot No. 55-116AF22; Product 2, Barr Lot No. 6C02013; Product 3, Ciba Lot No. 10721; Product 4, Danbury Lot No. 12357; Product 5, Heather Lot No. 61088; Product 6, Merck Sharp and Dohme Lot No. V2487; Product 7, Towne Paulsen Lot No. 037652; Product 8, Zenith Lot No. A208313.

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