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UPTAKE OF TAUROCHOLATE, A VECURONIUM-LIKE ORGANIC CATION, ORG 9426, AND OUABAIN INTO CARCINOGEN-INDUCED DIPLOID AND POLYPLOID HEPATOCYTES OBTAINED BY CENTRIFUGAL ELUTRIATION

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Abstract—Bile acid uptake, an important function of differentiated hepatocytes, is decreased in hepatocellular carcinomas and γ-glutamyltranspeptidase-positive, putatively preneoplastic hepatocytes. Whether hepatic uptake is also changed in carcinogen-induced diploid hepatocytes versus polyploid hepatocytes is unknown. The present study has determined whether the hepatic uptake of three model compounds, an anionic bile acid, an organic cation and a neutral organic compound, into diploid cells is different from that in polyploid hepatocytes. These two hepatocyte populations were separated from the parent freshly isolated hepatocyte suspension by centrifugal elutriation. Flow cytometric analysis indicated that the diploid fraction contained approximately 83% diploid cells and that the polyploid fraction had about 84% polyploid hepatocytes. Initial uptake velocity was determined for taurocholate (1–50 μM), ORG 9426 (20–400 μM), a vecuronium-like cation, and ouabain (20–500 μM). Apparent K_m was not different between diploid and polyploid cells for the three tested substrates, whereas apparent V_{max} was decreased in diploid hepatocytes for taurocholate and ouabain by 42 and 55%, respectively. There were no changes in the hepatic uptake of ORG 9426. These data indicate that uptake by the bile acid/multispecific carrier is compromised in carcinogen-induced diploid cells.

The liver efficiently removes from the blood many compounds ranging in size from small ions to complete cells. Although some chemicals enter hepatocytes by passive permeation of the lipid membrane, other compounds require more or less specific transport mechanisms including membrane carrier transport or receptor-mediated endocytosis [1–3]. Based on studies in isolated hepatocytes for the neutral compound ouabain [4, 5], for the organic anion bile acids [6–9], dibromosulphthalein [10], sulfobromophthalein and sulfobromophthalein–glutathione conjugate [11, 12], and for the organic cations procainamide ethobromide [13, 14] and vecuronium [15], hepatic uptake classically has been thought to include several carrier systems depending on the charge of the compound [11]. In recent years however, this established concept has been challenged as new data point to multispecific uptake systems [16–19].

Hepatic uptake of bile acids is an important function of the differentiated liver cell that is low at birth and matures during the first weeks after birth [20, 21]. This differentiated transport function appears to be lost during the process of transformation to a tumor cell. Thus, all hepatoma cells tested so far exhibit an almost total loss of carrier-mediated bile acid uptake [21–24]. The expression of the carrier may be affected already during the early stages of hepatocarcinogenesis. For example, it has been shown that hepatic uptake of cholate is decreased when rats have been chronically treated with diethylnitrosamine for a few weeks [25]. This may be partly due to the decreased transport activity of preneoplastic hepatocytes, i.e. of potential progeny of tumor cells. Indeed, γ-glutamyltranspeptidase-positive preneoplastic hepatocytes from 2-acetylaminofluorene (2-AAF‡)-treated rats exhibit a decrease in the apparent V_{max} for taurocholate uptake, whereas the apparent K_m was not changed [26]. Interestingly, the apparent V_{max} of bile acid transport has also been reported to decrease without changes in the apparent K_m in primary hepatocyte cultures, which are known to dedifferentiate [27]. Although these data indicated a partial loss of liver-specific function in preneoplastic hepatocytes, it is not known whether this effect is limited to only taurocholate or may be a generalized loss of carrier-mediated uptake for compounds of differing structure and charge.

In the present investigation we addressed the question of whether the uptake of neutral, anionic and cationic organic compounds is changed in small diploid hepatocytes representing another carcinogen-induced liver cell population. This cell population, which is induced by a distinct carcinogen regimen

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‡ Abbreviations: 2-AAF, 2-acetylamino fluorene; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycobis(aminoethylether)tetraacetate; MEM, minimal essential medium; DMEM, Dulbecco's MEM.

[28–31], most likely contains precursor cells of hepatocellular carcinomas [28, 32, 33]. Lines of evidence suggesting that these diploid cells may be involved in tumor development include: (1) the percentage of diploid hepatocytes is very low in the adult rat but increases markedly after treatment with carcinogens [28–31]; (2) the probability that a mutation will be expressed is much higher in a diploid than in a polyploid genome; and (3) diploid tumors have been found almost exclusively after treatment of rats with diethylnitrosamine plus 2-AAF or 2-AAF plus phenobarbital [28, 30, 33, 34]. In this context it has to be emphasized that adult rat liver is largely polyploid and that polyploidization most likely represents an irreversible aspect of hepatocellular differentiation [35].

It was the aim of the present investigation to study whether carcinogen-induced diploid hepatocytes have any alterations in the hepatic uptake process compared to "normal" polyploid liver cells. We therefore isolated diploid and polyploid hepatocytes from carcinogen-treated animals by centrifugal elutriation and determined the uptake of the bile acid anion taurocholate, a vercuronium-like cation, ORG 9426, and the neutral organic compound ouabain.

MATERIALS AND METHODS

Chemicals. Bovine pancreatic RNase A (90 U/mg), HEPES, ouabain octahydrate, polyvinylpyrrolidone (average M_r 40,000) and taurocholate, were obtained from Sigma Chemie (Deisenhofen, F.R.G.); EGTA and phenol reagent came from Merck (Darmstadt, F.R.G.); AR20 and AR200 silicone oils were purchased from Wacker Chemie (Munich, F.R.G.); bovine serum albumin and collagenase came from Boehringer Mannheim (Mannheim, F.R.G.); diethylnitrosamine and Dulbecco's minimal essential medium (DMEM) were purchased from Serva Feinbiochemika (Heidelberg, F.R.G.); and MEM amino acids and vitamins were from Biochrom KG (Berlin, F.R.G.). [^{3}H]Ouabain (15.4 Ci/mmol), [carboxyl-^{14}C]dextran (M_r 75,000, 1.3 mCi/g) and [^{3}H]water were purchased from NEN Chemie (Dreieichenhain, F.R.G.), and tauro-[carbonyl-^{14}C]cholic acid, sodium salt (50 mCi/mmol) was obtained from Amersham International (Amersham, U.K.). Radioactive and unlabeled ORG 9426 were generously provided by Dr. D. K. F. Meijer (University of Groningen, Groningen, The Netherlands). All other chemicals were of the highest quality commercially available. Double distilled water was used for all aqueous solutions.

Animals. Male Wistar rats approximately 4 weeks old (70–80 g) were obtained from the GSF breeding colony and had free access to food (standard pellet diet with or without 0.02% 2-AAF, Altromin, Lage, F.R.G.) and water throughout the experimental period. The rats were anesthetized with diethyl ether and a two-thirds partial hepatectomy was performed. Twenty hours later, diethylnitrosamine was administered by gavage at 50 mg/kg in water. One week after the partial hepatectomy, the rats were provided the standard pellet diet with 0.02% 2-AAF. After 4 weeks of 2-AAF feeding, the rats were switched to the standard diet without 2-AAF for 3–5 weeks before isolation of hepatocytes.

Isolation of hepatocytes. After anesthetization with sodium pentobarbital (100 mg/kg; 4 mL/kg in water), the abdominal cavity was opened by a midline incision, and the liver perfused via the portal vein with approximately 200 mL of Ca^{2+}-free modified Hank's solution containing 100 μM EGTA at a flow rate of 40 mL/min followed by 200 mL of the same Ca^{2+}-free medium without EGTA. Next, the liver was perfused for 10–15 min with 100 mL of DMEM containing 1.8 mM $CaCl_2$ and 0.12 U/mL of collagenase before Glisson's capsule was opened and the cells gently dispersed in DMEM. After filtration through 80 μm and subsequently 40 μm nylon mesh filters, the cells were washed three times in DMEM at 50 *g* for 45 sec. Viability, as determined by exclusion of 0.4% Trypan blue, was 90 ± 1%.

Centrifugal elutriation. Cells (125×10^6) were loaded into the Beckman JE-6B rotor at 1700 rpm, 8° and a flow rate of 19 mL/min. The medium contained: 137 mM $NaCl_2$, 5.6 mM KCl, 1 mM $MgSO_4 7H_2O$, 1 mM $CaCl_2 2H_2O$, 0.9 mM KH_2PO_4, 2.1 mM $Na_2HPO_4 2H_2O$, 20 mM HEPES, 5 mM glucose, MEM amino acids and vitamins, 1% polyvinylpyrrolidone, and 50 μg DNase I/mL, pH 7.4. The last two agents were included in the medium to protect the cells and improve flow conditions, and to prevent cell aggregation, respectively. Four 200-mL fractions were obtained by stepwise increments in flow rate at 1700 rpm: cellular debris and dead cells eluted between 19 and 28 mL/min, predominately diploid hepatocytes eluted between 28 and 34 mL/min, mixed fraction of diploid and polyploid cells at 34–50 mL/min, and finally the polyploid hepatocytes at 50–60 mL/min after decreasing to 1550 rpm. Cells in the diploid and polyploid fractions were concentrated by centrifugation in a Sorval RC-2B using the GSA rotor at 800 rpm for 45 sec. The medium was aspirated and the cells resuspended in the transport medium described below. Aliquots were removed for determination of cell number and viability as well as flow cytometry, and the remaining cells were used for the uptake studies.

Hepatic uptake. Aliquots of hepatocytes were diluted with appropriate volumes of transport medium, containing 132 mM NaCl, 5.2 mM KCl, 1 mM $MgSO_4 7H_2O$, 0.75 mM $CaCl_2$, 3 mM $NaH_2PO_4 H_2O$, 10 mM glucose, MEM amino acids and vitamins plus 2 mM L-glutamine and 15 mM HEPES, pH 7.4, to obtain a total volume of 1 or 2 mL. Then, 1×10^6/mL hepatocytes were incubated at 37° for 20 min before addition of 10 μL of radiolabeled substrates (15 nCi taurocholate, 37.5 nCi ouabain, 35 nCi vecuronium-like cation) in the following concentration ranges: 1–50 μM taurocholate, 20–400 μM ORG 9426 and 20–500 μM ouabain. Initial rates of uptake were determined by withdrawing 200-μL aliquots at 15, 30, 45, and 60 sec for taurocholate and 1, 2, 3, and 4 min for ORG 9426 and ouabain, layering the aqueous solution on top of 100 μL silicon oil (AR20/AR200 1:2) and immediately pelleting the cells by rapid centrifugation through the silicon oil into 3 M KOH [6]. All values were corrected for the amount of substrate in the

incubation medium adhering to the sedimented cells by determining separately the amount of [carboxyl-^{14}C]dextran pelleted with the cells. Radioactivity in the supernatants and in the cellular sediments was determined by liquid scintillation spectroscopy with automatic external standardization to account for quenching. To prevent chemoluminescence produced by the KOH, 50 μL of 3 M trichloroacetic acid were added to each vial to neutralize the KOH before addition of 4.5 mL of Beckman scintillation fluid. Apparent K_m and K_{max} were calculated from Hanes plots of the initial velocity data. The aqueous cell volume was measured using ^3H_2O and [carboxyl-^{14}C]dextran as described previously [6]. Cell protein was quantitated by the method of Lowry *et al.* [36].

Flow cytometric analysis. Cells were fixed by adding an equal volume of methanol (−20°) for 10 min. After centrifugation at 50 *g* for 1 min, the supernatant was removed and the cells were washed with phosphate-buffered saline and then resuspended in phosphate-buffered saline and gently mixed at 4° overnight. Then, the cells were incubated at 37° for 45 min with 200 μg heat inactivated RNase A. After addition of 60 μM propidium iodide, DNA content of hepatocytes in the parent cell suspension and elutriated fractions (diploid and polyploid) was determined using a FACS-Analyzer (Becton-Dickinson). Volume was determined by electrical resistance pulse sizing using a 75- or 100-μm orifice. DNA was routinely quantitated at 470–495 nm for excitation and 550–600 nm for emission in 10,000 events. The data were analysed using the Becton-Dickinson Consort 30 Program version E 12/86 and corrected for cell aggregates as described by Klose *et al.* [37].

Statistics. Means and standard errors were generated for all data, which were analysed by a one-way analysis of variance and Duncan's new multiple range test to compare the means. Significance was set at $P < 0.05$. Asterisks indicate that the values were significantly different from the parent suspension, whereas daggers indicate values for diploid hepatocytes were different from those for polyploid hepatocytes.

Table 1. Ploidy distributions of the parent hepatocyte suspension, and the diploid and polyploid fractions obtained by centrifugal elutriation

	% of total hepatocytes		
Fraction	Diploid	Tetraploid	Octaploid
Parent suspension	46 ± 2	49 ± 2	5 ± 1
Diploid	83 ± 2*	16 ± 3*	1 ± 1*
Polyploid	16 ± 3*	79 ± 3*	5 ± 1

Values represent means ± SE for six rats.
Asterisks indicate significant difference from the parent suspension at $P < 0.05$.

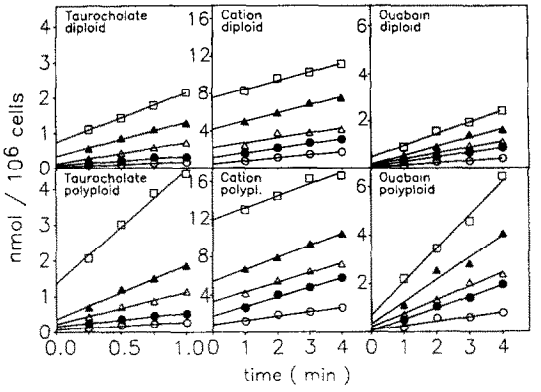

Fig. 1. Time-course for the initial uptake of taurocholate, ORG 9426, a vecuronium-like cation (cation), and ouabain by carcinogen-induced diploid and polyploid hepatocytes separated by centrifugal elutriation. At zero time, 10 μL of radioactive substrate were added to give the following final concentrations: (○) 1 μM taurocholate, (●) 2 μM taurocholate, (△) 5 μM taurocholate, (▲) 10 μM taurocholate, and (□) 50 μM taurocholate; (○) 20 μM cation, (●) 50 μM cation, (△) 100 μM cation, (▲) 200 μM cation; (□) 400 μM cation; (○) 20 μM ouabain, (●) 50 μM ouabain, (△) 100 μM ouabain, (▲) 200 μM ouabain, and (□) 500 μM ouabain. These data represent one of six determinations.

RESULTS

Sequential treatment of two-thirds hepatectomized Wistar rats with diethylnitrosamine and 2-AAF induced the emergence of diploid hepatocytes that were separated from polyploid cells by centrifugal elutriation. The purity of the elutriated cell fractions is indicated in Table 1 and was 83 and 84% for diploid and polyploid hepatocytes, respectively. Viabilities of the diploid and polyploid fractions determined immediately before initiation of the transport studies were 89 ± 3 and 93 ± 2, respectively, as measured with 0.4% Trypan blue. Cell protein content was significantly lower in diploid hepatocytes, being 0.49 ± 0.04 mg protein/10^6 cells vs 0.84 ± 0.08 mg protein/10^6 cells in the polyploid fraction.

Figure 1 illustrates the time-course for initial uptake of taurocholate, ORG 9426 and ouabain at different concentrations by diploid and polyploid hepatocytes. Uptake was linear for 1 min with taurocholate and 4 min for the other two substrates, and linearity was not affected by hepatocyte polyploidization. The initial rates of uptake were dependent on the concentration of the substrates.

A Hanes plot of these data from Fig. 1 yielded a straight line as shown in Fig. 2 and the apparent kinetic constants, K_m and V_{max}, were determined by linear regression analysis. Table 2 compiles the kinetic constants from the six separate experiments in diploid and polyploid hepatocytes. Values are expressed per milligram protein because of the differences in protein content per one million cells. It is clear that the apparent V_{max} for the uptake of taurocholate and ouabain was approximately 42 and 55% lower, respectively, in the diploid cell fraction than in the polyploid fraction. No change was observed in the apparent K_m value for the three substrates in either diploid or polyploid hepatocytes.

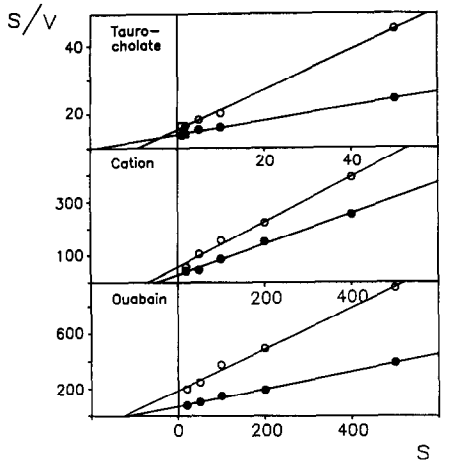

Fig. 2. Hanes plot of the initial uptake as a function of concentration for taurocholate, vecuronium-like cation and ouabain by carcinogen-induced diploid (○) and polyploid (●) hepatocytes separated by centrifugal elutriation. *S*, substrate concentration (μM); *V*, initial uptake rate (nmol/10^6 cells/min). Values are from one of six different determinations.

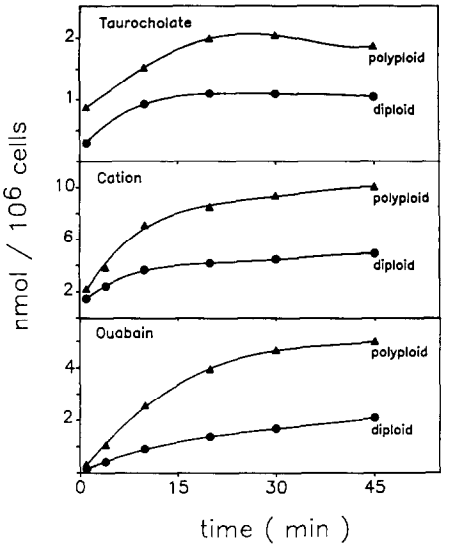

Fig. 3. Time-course for the uptake of 5 μM taurocholate, 50 μM vecuronium-like cation and 50 μM ouabain by carcinogen-induced diploid and polyploid hepatocytes separated by centrifugal elutriation. Values are one representative experiment out of three.

Table 2. Apparent kinetic constants for the uptake of taurocholate, ORG 9426 and ouabain in carcinogen-induced diploid and polyploid hepatocytes isolated by centrifugal elutriation

	Taurocholate		ORG 9426		Ouabain	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Diploid	13 ± 2	3.1 ± 0.5*	50 ± 3	1.8 ± 0.3	121 ± 13	0.9 ± 0.2*
Polyploid	16 ± 2	5.3 ± 1.0	39 ± 4	2.0 ± 0.2	143 ± 21	2.0 ± 0.3

Values for K_m (μM) and V_{max} (nmol/min/mg protein) represent the means ± SE for six determinations.

Asterisks indicate the diploid fraction is significantly different from the polyploid fraction at $P < 0.05$.

Figure 3 demonstrates that the uptake of radiolabeled substrate during a 45-min experimental period reached equilibrium between uptake, intracellular protein binding and excretion. Uptake reached a plateau at 20 min for taurocholate and somewhat later for ORG 9426 and ouabain. At 45 min, the amount of substrate at equilibrium between influx and efflux was 1.8-fold higher for taurocholate and more than 2-fold higher for ORG 9426 and ouabain in polyploid hepatocytes as compared to diploid cells. Mean values for the 45-min time points have been recalculated per milligram protein and are summarized in Table 3.

DISCUSSION

Sequential treatment of male Wistar rats with two-thirds partial hepatectomy, diethylnitrosamine and 2-AAF induces a population of diploid hepatocytes that is likely to contain the precursor cells of hepatocellular carcinomas [28, 33, 34]. The origin of this emerging liver cell population is still not known, but diploid hepatocytes could be derived from either oval cells, which must undergo transformation and then differentiation into hepatocytes [38–41], or a dividing diploid hepatocyte population [28, 32, 39]. Recent work in liver cells isolated from carcinogen-treated animals demonstrated that the capacity to take up the bile acid taurocholate was decreased in γ-glutamyltranspeptidase-positive putatively pre-neoplastic hepatocytes as compared to γ-glutamyl-transpeptidase-negative cells [26]. Similarly, γ-glutamyltranspeptidase-positive hepatocytes have an increased resistance to the mushroom poison phalloidin [42], whose uptake, a prerequisite for cytotoxicity, is likely to occur via the same carrier protein as taurocholate [17, 43]. The present investigation has determined whether diploid hepatocytes, another carcinogen-induced liver cell population [28–33], show a similar decrease in liver-specific transport processes.

Hepatocellular uptake was studied using three

Table 3. Equilibrium values for the uptake of taurocholate, ORG 9426 and ouabain in the parent hepatocyte suspension and in diploid and polyploid hepatocytes at 45 min

	Taurocholate	ORG 9426 (nmol/mg protein)	Ouabain
Diploid	2.50 ± 0.50	8.77 ± 1.84	4.08 ± 1.38
Polyploid	2.73 ± 0.33	12.54 ± 1.04	6.16 ± 0.91

Values represent means ± SE for three determinations.
The values for the diploid and polyploid cell fractions are not significantly different from each other at $P < 0.05$.

model compounds, the organic anion taurocholate, the organic cation ORG 9426 (a vecuronium-like compound) and the neutral organic compound ouabain. Transport of the three compounds was saturable and compatible with a carrier-mediated process.

The kinetic constants were expressed per milligram protein in Table 2 and not per 10^6 cells as in Figs 1 and 2. Carcinogen-induced diploid and polyploid hepatocytes differ in their mean diameters (17.3 and 22.6 μm, respectively) and cell volumes (2700 and 6000 μm^3, respectively) [44]. Due to their much larger size, transport will be higher in polyploid hepatocytes as compared to diploid cells when the data are expressed per million cells. Therefore, data must be expressed per surface area or per milligram protein. Because surface areas (940 and 1600 μm^2 for diploid and polyploid hepatocytes, respectively) and protein concentrations (0.49 ± 0.04 and 0.84 ± 0.08 mg protein/10^6 cells for diploid and polyploid hepatocytes, respectively) for the two liver cell fractions differ by a factor of approximately 1.7, data may be expressed either way without biasing the results.

The maximal velocity of transport was significantly lower in carcinogen-induced diploid hepatocytes as compared to the polyploid fraction for both taurocholate and ouabain, whereas apparent K_m values did not differ (Table 2). It appears that the number of transport sites for taurocholate and ouabain is decreased in carcinogen-induced diploid hepatocytes, but the affinity of the carrier is unchanged. In contrast, there was no change in the hepatic uptake of ORG 9426, which raises again the question of whether these compounds are transported by the same or different carrier proteins. During the last 10 years, increasing evidence has indicated that the liver has a sodium-independent transport system for organic anions such as sulfobromophthalein [11, 12, 45, 46] as well as a sodium-dependent carrier of broad substrate specificity. This multispecific transport system mediates uptake of bile acids, phalloidin, antamidine and possibly ouabain as indicated by both kinetic and photoaffinity labeling studies [4, 5, 17, 43, 47]. Our finding that both taurocholate and ouabain show a decreased V_{max} in carcinogen-induced diploid hepatocytes is compatible with the notion that the two compounds share a common carrier. On the other hand, uptake of ORG 9426 was similar in the two cell populations, suggesting a transport process distinct from taurocholate and ouabain. Other recent work has suggested that bivalent organic cations such as *d*-tubocurarine and vecuronium share the multispecific transport system of the liver [15, 48], and not the monovalent cation transporter. However, Meijer *et al.* [48] point out that it cannot be fully excluded that vecuronium also uses this alternative pathway for organic cations because K-strophantoside only partially affected the uptake of vecuronium. Our finding that the multispecific transport system of the liver is decreased in carcinogen-induced diploid hepatocytes indicates a partial loss of a differentiated liver cell function.

Figure 3 shows the marked accumulation of the three compounds in all liver cell fractions. Based on a water space of about 1.7 and 3.5 $\mu L/10^6$ cells in diploid and polyploid hepatocytes, respectively, which was determined with triated water, accumulation of taurocholate, ORG 9426 and ouabain was about 140-, 55- and 25-fold, respectively. There were no significant differences between the *accumulation* of the three compounds in the two cell populations (Table 3) despite the fact that *initial rates of uptake* of taurocholate and ouabain differ in the two cell populations (Table 2).

The present study has demonstrated that carcinogen-induced diploid hepatocytes exhibit a partial loss of a differentiated hepatocellular function. Specifically, there is a decrease in the maximal velocity for both taurocholate and ouabain transport into diploid hepatocytes, with no effects on carrier affinity for these substrates. These data suggest that taurocholate and ouabain are taken up into hepatocytes by the same carrier. Future work must address the questions: why is there a loss of transport function in diploid hepatocytes, and is the decreased uptake due to loss of carrier protein itself or due to diminished carrier function?

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