Partial Maintenance of Taurocholate Uptake by Adult Rat Hepatocytes Cultured in a Collagen Sandwich Configuration

Xingrong Liu,[1] Kim L. R. Brouwer,[1] Liang-Sheng L. Gan,[2] Kenneth R. Brouwer,[2] Bruno Stieger,[3] Peter J. Meier,[3] Kenneth L. Audus,[4] and Edward L. LeCluyse[1,5]

Purpose. This study was designed to characterize taurocholate uptake properties in primary cultures of rat hepatocytes maintained under different matrix conditions.

Methods. Hepatocytes isolated from male Wistar rats (230–280 g) were cultured on a simple collagen film, on a substratum of gelled collagen or between two layers of gelled collagen (sandwich configuration). Hepatocyte morphology, taurocholate uptake properties, and expression of the sinusoidal transport protein, Na^+/taurocholate-cotransporting polypeptide (Ntcp) were examined in these cultures at day 0 and day 5.

Results. By day 5, monolayer integrity had deteriorated in simple collagen cultures. In contrast, cell morphology was preserved in hepatocytes maintained in a sandwich configuration. At day 5, taurocholate accumulation at 5 min in hepatocytes cultured on a simple collagen film, on a substratum of gelled collagen, and in a sandwich configuration was ~13%, 20% and 35% of day-0 levels, respectively, and occurred predominately by a Na^+-dependent mechanism. The initial taurocholate uptake rate vs. concentration (1-200 μM) profile was best described by a combined Michaelis-Menten and first-order function. In all cases, the estimated apparent K_m values were comparable for day-0 and day-5 hepatocytes (32–41 μM). In contrast, the V_{max} values of hepatocytes cultured on a simple collagen film, on gelled collagen and in a sandwich configuration were ~5, 6 and 14% of the values at day 0, respectively; values for the first-order rate constant were 5-, 3- and 2-fold lower, respectively. Immunoblot analysis indicated that at day 5 Ntcp expression in hepatocytes cultured in a sandwich configuration was greater than in hepatocytes cultured on a simple collagen film.

Conclusions. A collagen sandwich configuration reestablishes normal morphology and partially restores bile acid uptake properties in primary cultures of rat hepatocytes.

[1] Division of Drug Delivery and Disposition, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599.

[2] Division of Bioanalysis and Drug Metabolism, Glaxo Wellcome, Inc., Research Triangle Park, North Carolina 27709.

[3] Division of Clinical Pharmacology, Department of Medicine, University Hospital, CH-8091 Zurich, Switzerland.

[4] Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66047.

[5] To whom correspondence should be addressed. (e-mail: ed_lecluyse@unc.edu)

ABBREVIATIONS: Ntcp, Na^+/taurocholate-cotransporting polypeptide; DMEM, Dulbecco's Modified Eagle's Medium; HBSS, Hanks' balanced salt solution.

INTRODUCTION

Primary cultures of hepatocytes currently are used in drug discovery and development to evaluate hepatic transport and metabolism of drugs, cytochrome P450 induction, drug interactions and drug-associated hepatoxicity (1). However, long-term cultured hepatocytes have not been used to study hepatobiliary transport due to a rapid loss of transport activities (2–5). For instance, the maximal taurocholate uptake capacity (i.e., V_{max}) was reduced to ~4% of day-0 levels in primary cultures of rat hepatocytes maintained for 72 hours under conventional culture conditions (6,7). Inasmuch as Na^+-dependent bile acid uptake represents a typical differentiated hepatocyte function, these observations agree well with the general concept that, under conventional culture conditions, primary cultures of rat hepatocytes dramatically lose many liver-specific properties and, therefore, are not a good model to study hepatobiliary transport (8,9).

This trend in the "de-differentiation" of cultured hepatocytes appears to be delayed by altering the biophysical state of the extracellular matrix. Several studies have shown that hepatocytes respond to a substratum composed of gelled collagen or Matrigel by expressing more liver-specific functions including bile acid synthesis while reducing the over-expression of "common genes" (e.g., actin) (10,11). Moreover, hepatocytes cultured between two layers of gelled collagen possess superior morphological and biochemical properties, such as albumin secretion, bile acid synthesis, and P450 enzyme induction, compared to cells on a single layer of gelled collagen (12–14). In particular, collagen-sandwiched hepatocytes develop an elaborate network of bile canaliculi that exhibit many normal structural and functional properties (13). Preliminary studies suggested that the measurement of substrates localized in the canalicular spaces may be utilized to predict *in vivo* biliary excretion of those substrates (15). However, a careful investigation of the effects of varying the geometry of the extracellular matrix on the retention of the bile acid transporters in primary cultures of rat hepatocytes has not been reported.

In this study, bile acid transport properties were characterized in primary cultures of rat hepatocytes maintained under different matrix conditions for up to 5 days. Taurocholate, a well-documented substrate of bile acid transporters, was employed as a model compound. Taurocholate enters hepatocytes predominantly through the Na^+/taurocholate-cotransporting polypeptide (Ntcp) and to a lesser extent by a Na^+/independent organic anion transporter (16).

Total cumulative taurocholate uptake, initial taurocholate uptake rate and cell morphology were assessed in 5-day-old cultures of hepatocytes maintained either on a simple collagen substratum (rigid collagen), on a single layer of gelled collagen or between two layers of gelled collagen (sandwich configuration). The expression of Ntcp in hepatocytes cultured on rigid collagen or in a sandwich configuration was assessed by immunoblot analysis.

MATERIALS AND METHODS

Chemicals. Taurocholate and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). [³H]Taurocholic acid (3.4 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). Collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and insulin were purchased from Gibco (Grand Island, NY). Rat tail collagen (type I) was obtained from Collaborative Biomedical Research (Bedford, MA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Animals. Male Wistar rats (250–280 g) from Charles River (Raleigh, NC) were used as liver donors. They were allowed free access to food and water, and were housed in a constant alternating 12-hr light and dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee.

Preparation of Culture Dishes. Plastic culture dishes (60 mm) were precoated with collagen, type I (0.1 ml, 1.5 mg/ml), in either a gelled or rigid state at least 1 day prior to preparing the hepatocytes (14). To obtain a simple (rigid) substratum, collagen solution was added to each dish. Coated dishes were stored in a sterile hood overnight. Immediately before use, fresh medium was added to neutralize the collagen. To obtain a gelled substratum, neutralized collagen was prepared and spread onto petri dishes. Freshly coated dishes were placed at 37°C in a humidified incubator for at least 60 min to allow the matrix material to gel, followed by addition of 3-ml fresh medium to the dishes and storage in a humidified incubator.

Isolation and Culture of Rat Hepatocytes. Hepatocytes were isolated from rats with a two-step perfusion modification of previously described methods (14). Hepatocyte viability was determined by trypan blue exclusion and was typically >90%. Cells were diluted with DMEM containing 5% fetal calf serum, insulin (4 mg/L), and dexamethasone (1 μM) and 2-ml aliquots were added to precoated dishes at a density of approximately 2×10^6 cells/60-mm dish. Approximately 1 hr after plating the cells, the medium was aspirated and replaced with fresh fully-supplemented DMEM. For hepatic transport studies, hepatocytes that had been seeded for 2–4 hr without collagen overlay were defined as day-0 or short-term cultured hepatocytes. To prepare cultures in a sandwich configuration, an ice-cold neutralized collagen solution (0.1 ml) was added to the top of cultures 24 hr after plating (14). These cultures were incubated 1 hour at 37°C to allow collagen to gel before adding 3-ml fresh medium to the dishes. Medium was changed on a daily basis until uptake studies were performed on the fifth day. These hepatocytes were referred to as day-5 or long-term cultured hepatocytes.

Taurocholate Uptake Studies. The influence of the matrix on non-specific binding by the tracer compound was determined after a series of washes with Hanks' balanced salt solution (HBSS). Dishes with a double-gel (without hepatocytes) or cultures in a sandwich configuration (with hepatocytes) were incubated for 10 min in 3 ml of 1 μM [³H]taurocholate in HBSS followed by sequential washes with 3-ml ice-cold HBSS.

Cells cultured in a sandwich configuration were lysed with 3-ml water after rinsing with 3-ml HBSS 9 times; thereafter washing with 3-ml HBSS was resumed.

Cultures were rinsed 2 times with 3-ml HBSS to remove incubation medium prior to transport studies. Each 60-mm dish received 3 ml of HBSS or choline buffer, in which sodium chloride was replaced with choline chloride on an equal molar basis, and was placed at either 4° or 37°C for 10 min before initiating experiments. Uptake was initiated by adding HBSS or choline buffer containing [³H]taurocholate to each dish at designated concentrations (see RESULTS). Incubations were carried out at 4 or 37°C for designated times. Taurocholate uptake was terminated by washing the cultures 4 times with ice-cold HBSS. After washing, 2 ml of 1% Triton X-100 solution was added to the culture dishes to lyse cells and an aliquot of lysate was analyzed by liquid scintillation spectrometry. Protein was measured with Bio-Rad *DC* Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Data in the plots are expressed as mean ± SD from 3 or 4 separate preparations of hepatocytes; triplicates were performed in each preparation, unless indicated otherwise. All values for taurocholate uptake into cell monolayers were corrected for nonspecific binding by subtracting taurocholate uptake determined in the appropriate control dishes in the absence of cells.

Immunoblot Analysis of Ntcp. Cells were collected by scraping into hypotonic lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 0.4 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10μg/ml leupeptin). The swollen cells were disrupted with 30 strokes in a tightly fitting Dounce homogenizer. The homogenate was centrifuged at $400 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $30,000 \times g$ for 30 min and the resulting pellet was used as the crude membrane fraction (18). Proteins from crude membrane fractions (50 μg) were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylaminde gel (Bio-Rad Laboratories, Hercules, CA) electrophoresis by the method of Laemmli (19). After proteins were transferred electrophoretically from SDS gels to nitrocellulose membranes (0.45 μm), the blots were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for 1 hr at room temperature. Rabbit anti-serum raised against a fusion protein containing the maltose-binding protein and the carboxyl-terminus of Ntcp was used as the primary antibody in the immunoblot analysis in the current studies (17). The blots were probed with polyclonal anti-Ntcp rabbit serum at 1:4000 dilution. Antibody binding was visualized with horseradish peroxidase-conjugated donkey anti-rabbit Immunoglobuline G serum at 1:2000 dilution, followed by detection with Amersham enhanced chemiluminescence kit and exposure on Amersham Hyperfilm according to the manufacturer's instructions.

Data Analysis. A combined Michaelis-Menten (K_m and V_{max}) and first-order function (K) was fit to the average of the initial rate of taurocholate uptake vs. concentration data to obtain estimates of the relevant kinetic parameters utilizing a nonlinear least-squares regression method (PCNONLIN, Version 3.0, SCI Software, Statistical Consultants, Inc., Lexington, KY). The criteria used to obtain the best model included Akaike's Information Criterion (20), the degree of co-linearity of parameters, the degree of bias in residual error, and visual

RESULTS

In order to examine accurately the kinetics of bile acid transport, it was essential to develop a wash procedure to remove most of the extracellular substrate efficiently after transport studies while leaving the intracellular substrate intact. Figure 1 represents the remaining taurocholate in a cell-free double-layer collagen gel and in hepatocyte cultures in a collagen sandwich configuration after each wash. During the first 4 washes, more than 99.8% of taurocholate was removed from the double-layer gel; 4 additional washes removed less than 0.2% of the original substrate. In collagen-sandwich cultures, the first 4 washes removed 74.0% of taurocholate; the next 4 washes only removed 0.7% of the original taurocholate. After the cells were lysed with water, 25.1% of taurocholate was removed by 4 washes and less than 0.2% of taurocholate remained in the cultures. These results indicated that 4 washes were adequate to remove most of the extracellular substrate while retaining the intracellular substrate.

Primary cultures of rat hepatocytes were maintained for 2–4 hours on either a layer of gelled collagen (Figure 2A) or on a rigid collagen substratum (Figure 2B) before conducting transport studies. Regardless of the substratum, hepatocytes began to show signs of cell spreading almost immediately after cell attachment. Hepatocytes maintained on a gelled collagen substratum displayed more compact spheroidal morphologies (Figure 2A). In contrast, hepatocytes maintained on a rigid collagen substratum spread and flattened more extensively (Figure 2B).

[3H]Taurocholate accumulation over time measured in the hepatocytes cultured on a rigid and gelled collagen for 2–4 hr is represented in Figure 3A and B, respectively. Considerable differences were observed between the passive (4°C) and active (37°C) components of taurocholate uptake for both matrix conditions. The non-specific, passive diffusion (4°C) was less than
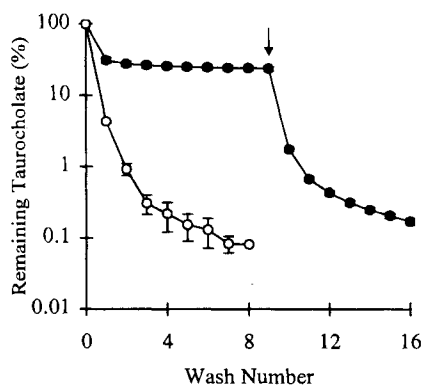

Fig. 1. Percent of radioisotope remaining vs. wash number in petri dishes containing a double-layer of collagen gel without hepatocytes (○) or a double-layer of collagen gel with hepatocytes (●). Dishes were incubated for 10 min in 3 ml of 1 μM [3H]taurocholate in HBSS followed by sequential washes with 3-ml HBSS. At the ninth wash (arrow), 3 ml of water was added to collagen sandwich cultures to lyse the cells; thereafter washing with 3-ml HBSS was resumed.
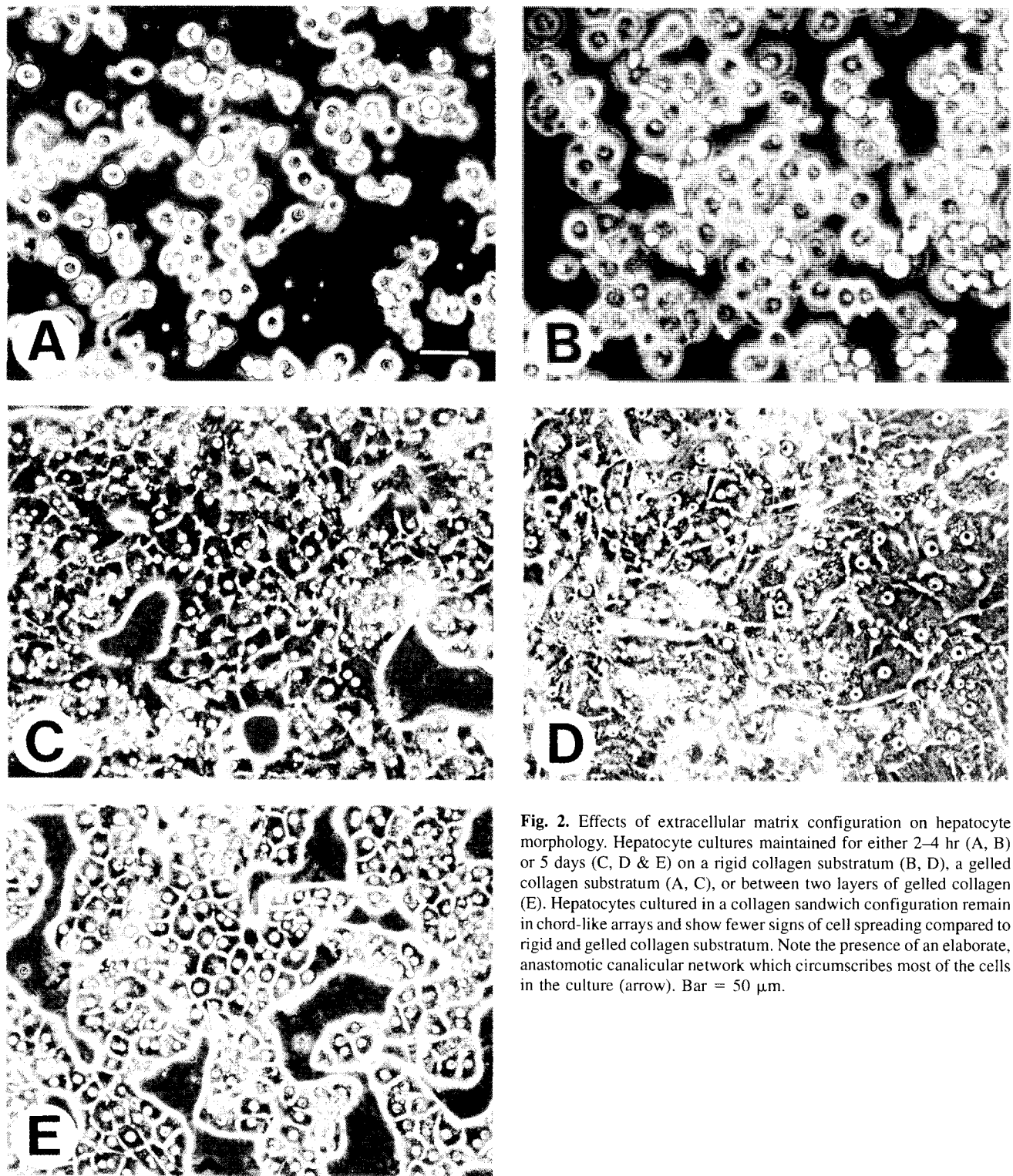
10% of the uptake at 37°C in both matrix conditions. Total taurocholate uptake in day-0 hepatocytes at 37°C was significantly higher in the cells grown on gelled collagen compared to rigid collagen. Taurocholate uptake was reduced by 70–80% in Na+-free buffer in the hepatocytes maintained on a rigid or gelled collagen substratum but the uptake was higher than the simple diffusion process (4°C; $p < 0.05$).

The second portion of this study was designed to examine taurocholate uptake properties in primary cultures of rat hepatocytes maintained on a rigid collagen substratum, on a gelled collagen substratum, or in a sandwich configuration for extended periods of time (5 days). Hepatocytes cultured on a rigid film continued to spread until a confluent monolayer was formed (Figure 2D). Although cultures of hepatocytes which had been maintained on gelled collagen for 5 days showed regions where the cells had reached confluence as was observed in cultures grown on a rigid film, cells on the gelled collagen remained in a more 3-dimensional state (Figure 2C). Under both matrix conditions without an overlay of collagen, hepatocytes typically exhibited significant spreading, a deteriorated morphology, and few signs of intact bile canalicular networks. In contrast, hepatocyte cultures maintained in a sandwich configuration (Figure 2E) remained in clusters or chords, exhibited less cell spreading, and displayed prominent networks of bile canaliculi compared to the other cultures.

Significant [3H]taurocholate uptake activity was maintained in hepatocytes cultured in a sandwich configuration at day 5 (Figure 3). The mean values of [3H]taurocholate at day-5 were greatest in the hepatocytes cultured in a sandwich configuration compared to on a rigid or gelled collagen substratum (Figure 3C-E; $p < 0.05$). For instance, on average at 5 min, hepatocytes cultured in a sandwich configuration retained ~35% of the original taurocholate uptake capacity of day-0 cultures while hepatocytes cultured on rigid and gelled collagen retained ~13 and 20%, respectively. The simple diffusion of [3H]taurocholate at 4°C was not significantly different among these cultures. Although the uptake of 1 μM [3H] taurocholate was reduced extensively in Na+-free buffer in collagen-sandwich cultures at day 5, it was still greater than the uptake at 4°C ($p < 0.05$).

Kinetic parameters describing taurocholate uptake were determined in hepatocytes cultured for 2–4 hr and hepatocytes cultured for 5 days on a rigid, or a gelled collagen substratum, or in a sandwich configuration. Taurocholate uptake vs. time appeared to be linear during the initial 60 seconds for all concentrations except 200 μM (Figure 4). Based on these results, initial rates of taurocholate uptake were determined between 15–30 seconds. A plot of the average initial rates of taurocholate uptake vs. substrate concentration (1–200 μM) from all culture configurations is illustrated in Figure 5.

Three kinetic models, first-order function, Michaelis-Menten process and Michaelis-Menten process in parallel with first-order function, were examined to select the best model to describe the initial uptake rates. Based on model selection criteria, a combined Michaelis-Menten and first-order function best described the initial uptake data using nonlinear least-squares regression analysis. Dissociation constants (K_m) and maximal rates of uptake (V_{max}) for the nonlinear uptake component and the first-order rate constant (K) for the linear uptake component were estimated from the mean data in Figure 5. Estimates of K_m were comparable for day-0 and day-5 cultures regardless

Fig. 2. Effects of extracellular matrix configuration on hepatocyte morphology. Hepatocyte cultures maintained for either 2–4 hr (A, B) or 5 days (C, D & E) on a rigid collagen substratum (B, D), a gelled collagen substratum (A, C), or between two layers of gelled collagen (E). Hepatocytes cultured in a collagen sandwich configuration remain in chord-like arrays and show fewer signs of cell spreading compared to rigid and gelled collagen substratum. Note the presence of an elaborate, anastomotic canalicular network which circumscribes most of the cells in the culture (arrow). Bar = 50 μm.

of the matrix configuration and ranged from 32–41 μM (Table 1). In contrast, day-5 hepatocytes cultured in a collagen-sandwich exhibited the greatest V_{max} value compared to hepatocytes cultured on rigid or gelled collagen for 5 days. The V_{max} of the hepatocytes cultured on rigid collagen, gelled collagen and in a sandwich configuration at day 5 was ∼5, 6 and 14% of the values at day 0, respectively. In addition, sandwich configuration and

gelled collagen conditions resulted in 2- to 3-fold lower first-order rate constant values for the linear portion of taurocholate uptake in day-5 cultures compared to that exhibited in day-0 cultures; however rigid collagen conditions resulted in a 5-fold lower rate for the linear portion of taurocholate uptake.

Immunoblot analysis showed that similar levels of Ntcp immunoreactive protein (51 kDa) are expressed in rat hepatocytes
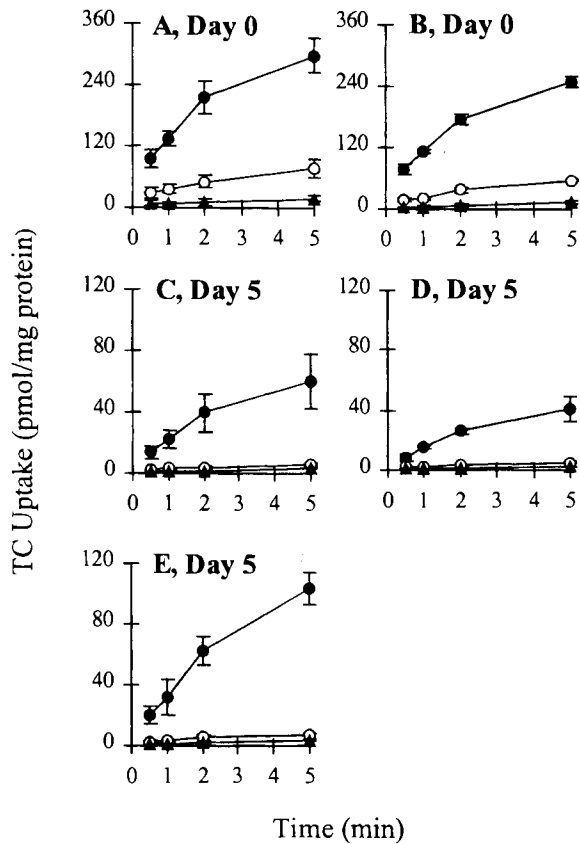
Fig. 3. Temperature- and Na^+-dependence of [³H]taurocholate (1 μM) uptake in cultured hepatocytes maintained under different matrix conditions as described in Figure 2 for either 2–4 hr (A, B) or 5 days (C, D & E) on a rigid collagen substratum (B, D), a gelled collagen substratum without an overlay of extracellular matrix (A, C), or between two layers of gelled collagen (E). Symbol ●, ○, and ▲ represents uptake in HBSS at 37°C, in Na^+-free HBSS at 37°C, and in HBSS at 4°C, respectively.

Fig. 5. Concentration-dependent initial rate of [³H]taurocholate uptake (in the presence of sodium) in day-0 hepatocytes cultured on a gelled collagen substratum (A, ●), in day-5 hepatocytes cultured in a sandwich configuration (B, ▲), day-5 hepatocytes cultured on a gelled collagen substratum (B, ■) and day-5 hepatocytes cultured on a rigid collagen substratum (B, ◆). Curved lines represent the fit of a combined Michaelis-Menten and first-order function to the data.

cultured on either a gelled or a rigid substratum for 2–4 hours (Figure 6, lanes 1 and 3). At day-5, Ntcp immunoreactive protein was detected in the hepatocytes cultured in a sandwich configuration (Figure 6, lane 2) but was undetectable in hepatocytes cultured on a rigid substratum (Figure 6, lane 4). After enhancement of

the sensitivity by an extended exposure time of the blot, the band intensity at 51 kDa increased significantly in the hepatocytes maintained in a sandwich configuration; however, Ntcp immunoreactive protein was not detectable in the hepatocytes cultured on a rigid collagen substratum (blots not shown).

DISCUSSION

The morphology of hepatocytes maintained in culture was drastically affected by the biophysical state of the extracellular matrix environment. Hepatocytes cultured in a collagen sandwich configuration displayed less cell spreading compared to rigid collagen and showed prominent networks of bile canaliculi throughout the culture period. This architectural conformation more closely resembled the cell plates, sinusoidal spaces and bile canaliculi observed in the intact liver.

Similarly, cumulative taurocholate uptake of hepatocytes maintained in culture also was affected significantly by the extracellular matrix environment. Taurocholate accumulation in hepatocytes cultured for a few hours on gelled collagen was greater compared to the cells on rigid collagen at 37°C while no difference was observed at 4°C. These observations indicated that the active transport process is maintained better in hepatocytes cultured on gelled collagen, but simple diffusion was
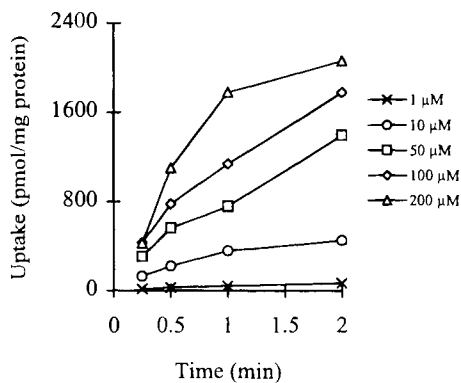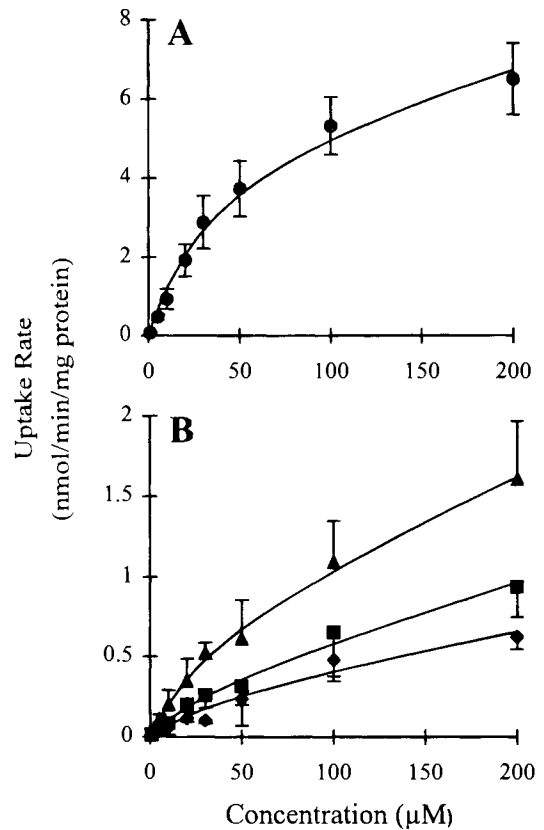

Fig. 4. Concentration-dependent cumulative [³H]taurocholate uptake vs. time in day-5 hepatocyte cultures maintained in a sandwich configuration (n = 2, range < 15% of average).

Table 1. Kinetic Parameters of Taurocholate Uptake in Cultured Hepatocytes

Culture condition	$K_m{}^a$ (μM)	$V_{max}{}^a$ (nmol · min^{-1} · mg protein^{-1})	K^a (10^{-3} · min^{-1} · mg protein^{-1})
Gelled Collagen (day 0)	36.9 ± 7.4	5.20 ± 0.39	3.85 ± 0.34
Sandwich Configuration (day 5)	35.8 ± 7.6	0.716 ± 0.056	1.69 ± 0.05
Gelled Collagen (day 5)	32.2 ± 15.9	0.291 ± 0.052	1.19 ± 0.05
Rigid Collagen (day 5)	40.6 ± 27.1	0.259 ± 0.061	0.74 ± 0.05

[a] Parameters (mean ± SE, n = 6–9) were obtained by fitting a Michaelis-Menten and a first-order function to the data in Fig. 5 with nonlinear least-squares regression analysis.

essentially the same. These data confirm previously reported results that transport capacity of cultured hepatocytes deteriorates rapidly under conventional culture conditions (2). Taurocholate accumulation deteriorated with time in hepatocytes maintained in culture for up to 5 days. However, this decrease in transport capacity was attenuated by the addition of a collagen gel substratum and overlay. Collagen-sandwich cultures at day 5 retained as much as 35% of the cumulative uptake (5 min) of day-0 cultures. Further studies have indicated that cumulative uptake of taurocholate in sandwich-cultured hepatocytes decreases gradually. After the hepatocytes were maintained in a sandwich configuration for 72 hr, cumulative taurocholate uptake was 63% of the uptake in the hepatocytes cultured for 2–4 hr. In contrast, previous studies reported that hepatocytes maintained under conventional conditions only retain 2–10% of cumulative uptake at 72 hr (2,3). Thus, primary rat hepatocytes cultured in a collagen-sandwich configuration retain significant taurocholate cumulative uptake for a prolonged period of time.
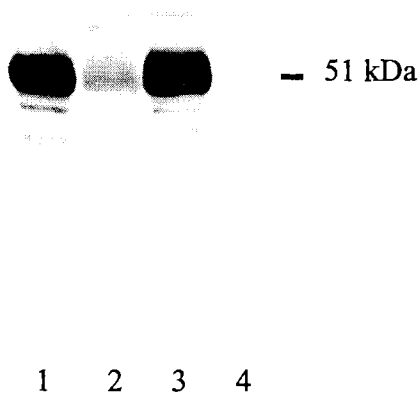

Fig. 6. Immunoblot analysis of Ntcp in primary cultured rat hepatocytes. All lanes were loaded with 50 μg crude membrane protein isolated from cultured hepatocytes. Lanes 1 and 3 were from hepatocytes cultured for 2–4 hr on a gelled collagen substratum and on a rigid collagen substratum, respectively. Lanes 2 and 4 were from day-5 hepatocytes cultured in a sandwich configuration and on a rigid collagen substratum, respectively. Ntcp, a single broad band at 51 kDa was detected in hepatocytes cultured for 2–4 hr or hepatocytes cultured in a sandwich configuration for 5 days, but was not detected in hepatocytes cultured on a rigid collagen substratum for 5 days.

Maintenance of the expression of transport proteins such as Ntcp in long-term cultured hepatocytes was affected significantly by the extracellular matrix. Western blot analysis demonstrated the presence of immunoreactive Ntcp protein (molecular mass 51 kDa) in crude membranes isolated from cultured hepatocytes. After prolonged exposure, the intensity of the Ntcp band was enhanced markedly in hepatocytes cultured in a sandwich configuration, but could not be detected in hepatocytes cultured on a rigid collagen substratum. These results show that Ntcp was maintained partially in the hepatocytes cultured in a collagen-sandwich configuration but was lost completely in hepatocytes cultured on a rigid collagen substratum at day 5. These differences in taurocholate uptake properties observed between hepatocytes cultured under different extracellular matrix conditions may be attributed to greater levels of Ntcp expression in hepatocytes cultured in collagen-sandwich configuration compared to hepatocytes cultured on rigid gelled collagen.

Liang, *et al.* (2) demonstrated a parallel reduction of the Na$^+$-dependent taurocholate uptake capacity and the Ntcp-encoding mRNA levels with increasing culture times. Hence, down regulation of the Ntcp gene appears to be the primary cause of incompetent Na$^+$-dependent bile acid uptake in cultured rat hepatocytes. In this study, hepatocytes cultured in a collagen-sandwich configuration maintained taurocholate uptake properties and Ntcp, suggesting that the collagen-sandwich configuration may be able to restore partially Ntcp gene expression and/ or to stabilize Ntcp protein.

Taurocholate uptake in sandwich cultures occurred mainly by a Na$^+$-dependent mechanism and to a lesser extent by a Na$^+$-independent mechanism in both hepatocytes cultured for 2–4 hours on gelled collagen and for 5 days in a sandwich configuration. These results are consistent with published data indicating that taurocholate uptake *in vivo* occurs predominantly by a Na$^+$-dependent mechanism (16). Cultured hepatocytes treated with Na$^+$-free buffer showed a 70–80% reduction at day 0 but 90–95% reduction at day 5 in the total cumulative taurocholate uptake compared to untreated cultures. These results suggest that the Na$^+$-independent component of taurocholate uptake deteriorates more rapidly than the Na$^+$-dependent component.

The mean K_m values for hepatocytes cultured under all conditions were similar, however, estimates for V_{max} in hepatocytes cultured on rigid collagen, gelled collagen and in a sandwich configuration at day 5 were 5, 6 and 14% of the values at day-0, respectively. These findings are in agreement with reports for taurocholate uptake in hepatocytes indicating that K_m values remain relatively stable over time in culture, but V_{max} progressively declines (2). In sandwich-cultured hepatocytes, the V_{max} for initial uptake and the 5-min cumulative

uptake were 14% and 35% of the respective values in 2–4 hr cultured hepatocytes. This apparent discrepancy is probably due to the absence versus presence of a bile canalicular compartment in short-term and long-term cultured hepatocytes, respectively, resulting in greater apparent cumulative uptake capacity in long-term cultured hepatocytes. Interestingly, the first-order rate constant for taurocholate uptake at day 5 was 5-, 3- and 2-fold lower than the values at day 0. It has yet to be determined whether the reduction in the rate constant for the linear portion of taurocholate uptake in hepatocytes cultured under different extracellular matrix conditions reflects deterioration in one of the taurocholate transporters (i.e., Na^+-independent) or the inability of one transporter to function effectively.

Inasmuch as hepatocytes cultured in a sandwich configuration form extensive bile canalicular networks and partially maintain bile acid uptake function, this *in vitro* model may have several applications in drug discovery and development. For instance, preliminary studies suggested that endogenous and exogenous compounds can accumulate in the canalicular networks of primary hepatocytes cultured in a sandwich configuration for 5 days. Canalicular substrate accumulation may be quantitated by modulating Ca^{2+} in the incubation buffers (15). Thus, as Caco-2 cell monolayers are used to study drug absorption properties, this *in vitro* model also may have utility as a screening tool to identify the extent of biliary excretion of drug candidates *in vivo*.

In summary, this study has demonstrated that hepatocytes cultured in a collagen sandwich configuration retain superior bile acid transport properties over time compared to hepatocytes cultured without a collagen overlay. Hepatocytes cultured in a sandwich configuration may be a useful model for *in vitro* studies of the expression and regulation of hepatobiliary transport function.

ACKNOWLEDGMENTS

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