Studies of bile acid secretion by isolated rat hepatocytes

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Abstract Bile acid secretion was studied in isolated rat hepatocytes cultured in medium supplemented with cofactors and succinate. Succinate as a respiratory substrate was found to be essential for bile acid synthesis. Feedback mechanism was demonstrated with different inhibitory potency of different bile acids on bile acid synthesis in isolated hepatocytes.

Supplementary key words bile acid synthesis · hepatocyte culture

Studies of isolated cells from different organs afford an opportunity to examine the metabolism of intact cells that are free from the influence of circulating hormones and metabolites found in the intact animal but that retain a greater degree of integrity and physiological organization than homogenates and cell fragment systems. The principal disadvantages stem from damage to the plasma membrane incurred during cell separation and ultrastructural changes which vary in degree with the gentleness of the method selected for cell preparation.

Recently, isolated hepatocytes have been used to study the metabolic activities of the liver at a cellular level (1–8) including metabolism of bile acid secretion in isolated rat hepatocytes. Such studies could provide a suitable experimental model that reflects metabolic disorders or the effect of different drugs on bile acid or cholesterol synthesis at a cellular level. Our studies were designed to evaluate the effect of different media and cofactors on bile acid secretion in isolated hepatocyte cultures. In addition, the effect of primary bile acids on bile acid synthesis and secretion patterns was investigated.

MATERIALS AND METHODS

Male Wistar rats, 250–300 g, that were fed or fasted for 18 hr were used for this study. Rats were maintained on Purina standard diet with water and kept in the dark overnight.

Preparation of rat liver hepatocytes

The parenchymal cells were prepared by a modification of the technique of Bonney (9). The liver was perfused for 15–20 min at a rate of 6–7 ml/min using a polystaltic pump with Ca^{2+}-free Hank's solution containing 0.2 g% bovine albumin. Perfusion at a rate of 6–7 ml/min was continued for an additional 12–15 min period with 0.05% collagenase (obtained from Sigma Chemical Co., St. Louis, MO) in Ca^{2+}-free Hank's solution containing 0.2 g% bovine albumin (Fraction V obtained from Sigma Co.). All the solutions were sterilized by filtration and kept at room temperature. They were gassed with a 95% O_2 and 5% CO_2 mixture during the perfusion. After the enzyme perfusion, the liver was separated as a bag of cells requiring very light mechanical treatment to separate the hepatocytes in a few ml of diluted enzyme solution at room temperature. The mixture was diluted with Hank's solution filtered through gauze and then through 88-μm mesh nylon gauze into a plastic centrifuge tube. The cells were washed with Hank's solution and centrifuged twice at 50 *g* for 2 min and once at 100 *g* for 5 min. The final pellet was suspended in Tris-KCl buffer (containing 20 mM Tris-HCl and 100 mM KCl, pH adjusted to 7.4 with HCl) and used for inoculating, counting, wet weight, and protein determinations. Under the best conditions, the average cell yield was 2.5–4 g of wet cells per liver with over 95% viability as shown by trypan blue exclusion.

Incubation of liver cells

One to two ml of suspension containing 200–300 mg of cells was incubated in 50-ml siliconized Erlenmeyer flasks in a total volume of 9 ml of the different media under investigation. In some experiments one

Abbreviations: TC, taurocholate; CA, cholate; CDC, chenodeoxycholate; BB biliary bile; HB, hepatocyte bile; HSB, hepatocyte secreted bile.

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volume of cell suspension in Tris-KCl and one volume of stock medium (double strength) were mixed before incubation to furnish the required culture medium. The flasks were incubated at 37°C with continuous shaking (90 oscillations/min with an amplitude of 4 cm). The flasks were aerated intermittently by a stream of 95% O_2 and 5% CO_2, the pH was checked, and aliquots of cells were stained with trypan blue. Viability was over 90% at the end of the incubation period of 3 hr.

Culture Media

The following media were investigated.

Medium 1. Hank's solution containing 0.2 g% bovine albumin and 1 mg/ml penicillin.

Medium 2. RPMI-1640 in 25 mM HEPES buffer with glutamine (obtained from Grand Island Biological Company, Grand Island, N.Y.) supplemented with 1 mg/ml penicillin.

Medium 3. Tris-KCl containing 0.2% albumin, 0.1 mM $MnCl_2 \cdot 4H_2O$, 3.3 mM $MgCl_2 \cdot 6H_2O$, 3.3 mM sodium citrate, 10 mM sodium succinate, 0.03 mM coenzyme A, 6.7 mM nicotinamide, 1.7 mM glucose-6-phosphate, and 1 mg/ml penicillin.

Bile acid determination

At intervals of incubation time, 1 ml of incubated cell suspension was centrifuged immediately at 3,000 rpm for 5 min. The supernatant was frozen. Total bile acids were obtained enzymatically with hydroxysteroid dehydrogenase by a modification of the method of Small and Rapo (10) as previously reported by Dennis et al. (11). Samples of biles were diluted 1:10 with methanol and 0.1 ml was used for the assay. For assay of bile acids in the incubating medium, 0.1 ml was diluted with methanol, 1:1, and, was used after centrifugation. All determinations were performed in duplicate and a set of standards was always assayed with each batch of tests. In preliminary experiments, recoveries of added bile acids were greater than 96%. The data represent the mean of results obtained from hepatocytes isolated from three to four different rats. For comparison of different media, the same liver preparation was used with each medium tested.

Individual bile acids in biles, liver cells, and medium after methanol–acetone extraction were separated on silica gel G plates developed with the appropriate solvent and quantitatively determined according to Dennis et al. (11).

Biochemical assays

The net production of glucose, lactate, and pyruvate during different intervals of the 3-hr incubation period was determined enzymatically by subtracting the zero-time amount and using the method of Boehringer Mannheim Ltd. (12). Lactic dehydrogenase was determined in cells after homogenization in phosphate buffer of pH 7.4 using the method of Boehringer Mannheim Ltd. (12). Protein content of hepatocytes was determined by the method of Lowry et al. (13).

RESULTS AND DISCUSSION

The modification of Bonney's technique (9), which has been adopted in this laboratory for hepatocyte isolation, yielded a nine to tenfold greater harvest of intact whole cells than that of Howard, Lee, and Pesch (14). Under optimal conditions, $20–25 \times 10^6$ cells were recovered per gram of liver. These preparations were not greatly contaminated by blood cells, as shown under the microscope, and the viability was over 95%. The availability of a large number of cells from a single liver provides a further advantage in these metabolic studies. Bonney (9) showed that the short-term incubation of isolated hepatocytes in a completely defined medium yields a homogenous cell population that exhibits several of the in vivo functions. The integrity and metabolic state of the cells after 3 hr of incubation were evaluated by trypan blue exclusion, lactate–pyruvate ratio, glucose production rate, and retention of lactate dehydrogenase (LDH). It was found that trypan blue exclusion was over 90% and the lactate–pyruvate ratio ranged from 7.5 to 9.5. The average glucose production was 12.9 μmol/g hepatocytes per hr. Hepatocytes retained over 85% of the LDH activity which dropped from 3,400 mU LDH at zero time to 2,900 mU LDH per g hepatocytes at the end of the 3-hr incubation period.

Incubation media

Evaluation of different media for enhancing bile acid synthesis is a significant step towards studying the metabolic activity of hepatocytes for bile secretion. **Table 1** shows the rate of bile acid secretion at different time intervals by hepatocytes from fasted rats when the cells were suspended in different media. The data show that the secretion rate of bile acids by hepatocytes suspended in Hank's solution or RPMI-1640 medium was very low, although RPMI-1640 contains almost all the amino acids, vitamins, minerals, and 2.0 g of glucose/l. Supplementation with citrate, trace elements, substrates, and cofactors found essential for lipid synthesis by subcellular fractions of liver and liver hepatocytes (1, 15) markedly increased

the secretion rate of bile acids. This indicates that liver cells may have been depleted of factors essential for bile acid synthesis during cell isolation. The bile acid secretion was linear for 1 hr and then became stable until the end of the incubation period. This may reflect the equilibrium stage or may result from total inhibition of both secretion and the re-uptake process.

Cofactor requirements

A negligible amount of bile acids was secreted by hepatocytes incubated in Tris-KCl solution. Therefore, a number of cofactors found essential for lipid synthesis by subcellular fractions of liver (15) and for lipid synthesis by hepatocytes (1) were tested in similar concentrations for their effect on bile acid secretion. The results presented in **Tables 2** and **3** show that the combination of a few factors is essential for bile acid secretion. Omission of Mn^{+2} and citrate induced bile acid secretion while omission of succinate was found to be detrimental. Supplementing Tris-KCl solution with each component separately shows that succinate is particularly essential for bile acid synthesis. Acting separately, none of the components was found to maintain the secretion rate at as high a level as the combined components. CoA and glucose 6-phosphate had no effect on secretion of bile acids by hepatocytes, although Cappuzi and Margolis (1) reported the detrimental effect of the absence of both on [^{14}C]acetate incorporation into lipid fractions. This may indicate that these two factors did not leak or penetrate from or through the cell membrane of hepatocytes isolated by our method. In our method of isolation, very light mechanical trauma was used to segregate the cells, which may be reflected in little damage to the cell membranes. However, the degree of leakage and membrane permeability depends on the method used for hepatocyte isolation (2). This does not exclude an ultrastructural change for the cellular membrane. The loss of soluble glycolytic enzymes that might occur during cell isolation impairs endogenous respiration by hepatocytes and prevents the utilization of glucose (16). However, because of particular mitrochondrial enzymes that are retained, the addition of succinate, fumarate, and malate markedly stimulate cellular oxygen consumption (17) and probably result in enhancement of bile acid synthesis by their utilization as respiratory substrates. The same observation was previously reported for using succinate in lipid synthesis by isolated hepatocytes (1). The selective permeability of isolated hepatocytes for different substrates is made clear by replacing succinate with different respiratory substrates after omitting citrate, $MnCl_2 \cdot 4H_2O$ and CoA from the medium (**Table 4**). Only dicarboxylic acids of the tricarboxylic acid cycle can be utilized as respiratory substrates. This may be due to the similarity in the stereoconfiguration that allows these acids to penetrate the cell membrane.

TABLE 1. Bile acid secretion by rat hepatocytes[a] in different media

Time	Med. 1[b]	Med. 2[c]	Med. 3[d]
hr	*nmol/mg protein ± S.E.*	*nmol/mg protein ± S.E.*	*nmol/mg protein ± S.E.*
0	0	0	0
0.25	11.8 ± 0.3	11.8 ± 0.9	661.0 ± 18.2
0.5	14.2 ± 0.1	16.5 ± 0.3	807.7 ± 5.2
1	26.0 ± 0.8	28.3 ± 1.4	916.4 ± 5.0
2	33.0 ± 1.1	28.3 ± 2.3	824.3 ± 1.4
3	52.0 ± 2.8	30.7 ± 1.7	781.8 ± 15.1

[a] Hepatocytes were isolated from fasted rats and cultured in three different media.
[b] Hank's solution containing 0.2 g% bovine albumin and 1 mg/ml penicillin.
[c] RPMI-1640 in 25 mM HEPES buffer with 1 mg/ml penicillin.
[d] Tris-KCl containing 0.2 g% albumin, 0.1 mM $MnCl_2$, 3.3 mM $MgCl_2$, 3.3 mM sodium citrate, 10 mM sodium succinate, 0.03 mM co-enzyme A, 6.7 mM nicotinamide, 1.7 mM glucose-6-phosphate and 1 mg penicillin.

TABLE 2. Cofactor requirements for bile acid secretion

Cofactor Components	Concn. mM	Secretion Rate (at 2 hr) (nmol/mg protein ± S.E.)
Complete mixture[a]		1402.9 ± 38.0
Tris-KCl alone		23.6 ± 4.3
$MgCl_2 \cdot 6H_2O$ (Tris-KCl)	3.3	27.0 ± 2.7
$MnCl_2 \cdot 4H_2O$ (Tris-KCl)	0.1	35.0 ± 2.8
Succinate (Tris-KCl)	10	1039.2 ± 21.6
Citrate (Tris-KCl)	3.3	146.4 ± 2.5
Nicotinamide (Tris-KCl)	6.7	40.8 ± 2.1
CoA (Tris-KCl)	0.03	47.8 ± 2.0
Glucose-6-phosphate (Tris-KCl)	1.7	41.5 ± 2.1

[a] Medium 3. Each component was supplemented separately in Tris-KCl solution to furnish the required concentration.

TABLE 3. Effect of omitting different components of supplemented medium (Med. 3) on bile acid secretion

Component Omitted	Secretion Rate (at 2 hr) (nmol/mg protein) ± S.E.
All components	21.3 ± 0.9
None	1006.1 ± 5.8
$MgCl_2 \cdot 6H_2O$	1091.2 ± 16.3
$MnCl_2 \cdot 4H_2O$	1242.3 ± 0.9
Succinate	23.6 ± 2.2
Citrate	1204.5 ± 2.5
Nicotinamide	1060.5 ± 3.7
CoA	1069.9 ± 1.6
Glucose-6-phosphate	975.4 ± 16.8

In these experiments each of the components of medium 3 was omitted separately and the media were tested in the presence of all the components except the omitted ones.

TABLE 4. Secretion of bile acids in the presence of various substrates of the tricarboxylic acid cycle[a]

Substrate	Secretion Rate (at 2 hr) (nmol/mg protein) ± S.E.
Succinate	1599.0 ± 10.4
Lactate (Li salt)	193.7 ± 10.4
Fumarate	2411.4 ± 1.4
Malate	2213.0 ± 44.0
α-Ketoglutarate	175.5 ± 3.9
Pyruvate	73.2 ± 5.5
Citrate	153.5 ± 11.8

[a] In these experiments citrate, $MnCl_2 \cdot 4H_2O$ and CoA were excluded from medium 3. Succinate was replaced with each of these substrates at a concentration of 10 mM and pH was adjusted to 7.4.

Effect of primary bile acids on bile acid secretion by rat hepatocytes

The effects of different concentrations of cholate, chenodeoxycholate, and taurocholate on bile acid secretion are shown in **Fig. 1.** It is obvious that chenodeoxycholate has a stronger inhibitory potency (92%) on bile acid synthesis than taurocholate or cholate (61% and 46%, respectively). Lower concentrations of bile acids have either no or a negligible inhibitory effect. A study of the time course of bile acid secretion in the presence of 3.3 mM of primary bile acids (**Fig. 2**) shows that secretion increased linearly with incubation time in the presence of taurocholate and cholate. In the presence of chenodeoxycholate the bile acid secretion increased and then attained a plateau after 10 min, reflecting an equilibrium between influx and efflux.

This report is the first demonstration of the feedback mechanism in bile acid synthesis using isolated hepatocytes. The feedback mechanism was previously observed in isolated rat and dog livers (18, 19). Our in vitro study of bile acid secretion by hepatocytes supports the idea that production of bile acids by hepatocytes is regulated by a feedback mechanism similar to that of cholesterol biosynthesis. Cooper and Margolis (20) observed the feedback mechanism in lipid synthesis in isolated rat hepatocytes.

In most mammals, cholesterol is converted to the two primary bile acids, cholic and chenodeoxycholic acids. These bile acids have similar although not identical properties (19), but only recently have a few reports presented the differences in the physical and pharmacological properties, Anwer, Kroker, and Hegner (21) found that different bile acids inhibited the uptake of both cholate and taurocholate and their relative inhibitory potency was not the same for both bile acids. In another report (6) taurocholate uptake was shown to be competitively inhibited by taurochenodeoxycholate in rat hepatocytes. Intestinal infusion of taurochenodeoxycholate into rats with bile fistulas inhibited taurocholate production (22). However, the difference in the inhibitory potency of chenodeoxycholate and cholates can be explained on the basis of the following possibilities.

1) There could be different carriers for chenodeoxycholate and cholate. A rapid adsorption for CDC carrier to the lipid phase of the plasma membrane leads to the inhibition of taurocholate faster than TC or CA carrier. There is some indirect evidence to support the different carrier hypothesis for the different bile acids (6, 21, 23).

2) Bile acids are transported by a common carrier, but each bile acid has a different binding affinity to the carrier (19). A common carrier hypothesis is supported by the evidence that taurocholate is competitively inhibited by chenodeoxycholate in isolated rat hepatocytes (6) and dog liver (24).

Because the secretion of TC is competitively inhibited by CDC and TC is the major component of rat biles (78%), the inhibitory potency of CDC is more obvious than that of cholate or TC. This finding agrees with the previous observation that chenodeoxycholic acid is more potent at inhibiting hydroxymethylglutaryl-CoA reductase or its synthesis than

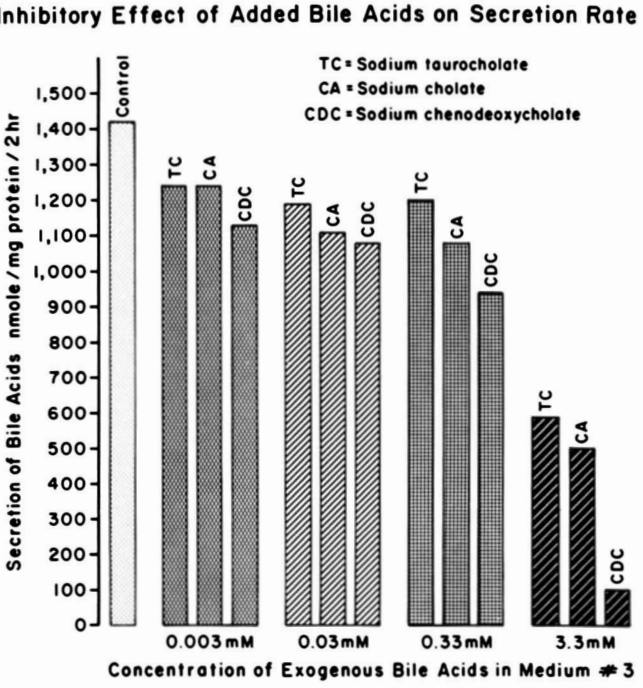

Fig. 1. In these experiments hepatocytes (isolated from fed animals) were cultured in medium 3 (omitting citrate, $MnCl_2 \cdot 4H_2O$, and CoA), supplemented with different bile acids to furnish the required concentration. After 120 min of incubation, bile acids were assayed enzymatically in the culture medium and secreted bile acids were calculated. There is a significant depression in bile acid secretion from hepatocytes when bile acids were supplemented to the medium.

cholic acid (25). However, it was found that the amount of secreted bile acids in the first hour of the control experiment (average 9 μmol/3 ml culture medium) is almost equivalent to the amount of exogenous CDC added (10 μmol/3 ml culture medium), which may explain the plateau effect observed.

Comparative study of bile acid pattern in biliary bile (BB), hepatocytes (HB), and hepatocyte secreted bile (HSB)

Bile acids of BB, HB, and HSB were fractionated on silica gel, identified, and quantitatively determined.

The analyses of these biles revealed the presence of different bile acids in different concentrations. In hepatocytes incubated for 60 min, there were two unidentified bile acids that were not detected in either BB or HSB. One of these acids was located between taurochenodeoxycholic and glycocholic acids and the other unidentified acid was detected between glycocholic and glycochenodeoxycholic acids. Although Anwer et al. (5) did not detect free bile acids in their culture medium, we were able to detect trace amounts of chenodeoxycholic acid under these conditions. The free bile acids detected in both BB and HSB were different; cholic acid was present in BB and chenodeoxycholic acid was present in HSB.

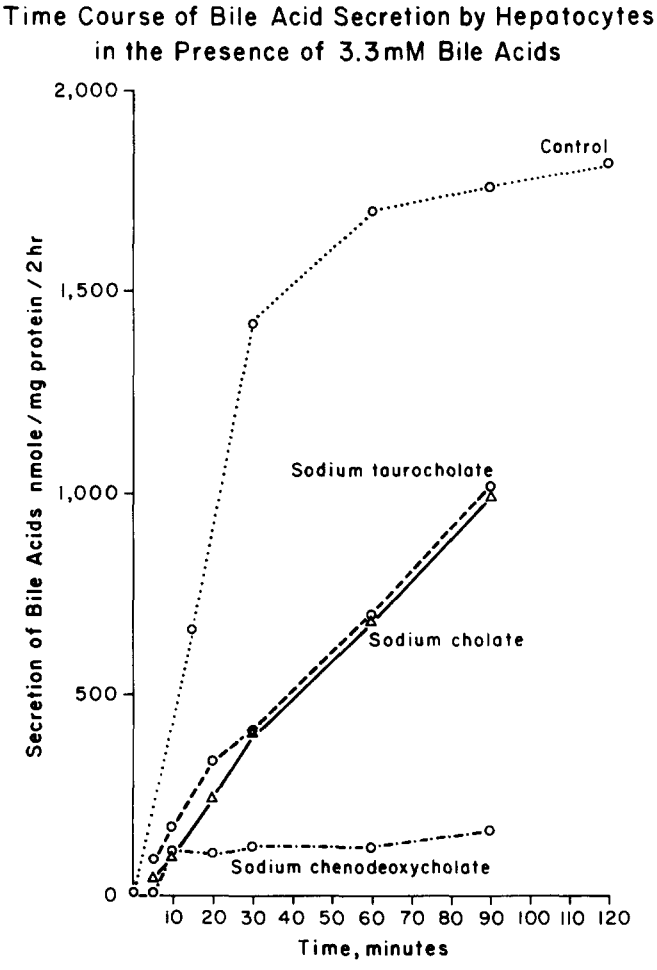

Fig. 2. In these experiments different bile acids were supplemented to the cultured hepatocytes (as in Fig. 1) to furnish a concentration of 3.3 mM. At different time intervals, samples were taken from the cultured hepatocytes and used for bile assay in the medium. The secreted bile acids were then calculated. It is clear that 3.3 mM concentration of chenodeoxycholate has a significant depressing effect on bile acid secretion when compared to cholate or taurocholate.

TABLE 5. Bile acids distribution in biliary bile (BB),[a] hepatocytes (HB)[b] and hepatocyte secreted bile (HSB)[c]

	BB %	HB %	HSB %
Taurocholic	69.4	42.4	29.9
Taurochenodeoxycholic	13.4	9.5	12.7
Unknown		8.0	
Glycocholic	8.5	15.1	25.3
Unknown		10.3	
Glycochenodeoxycholic	6.2	7.5	25.3
Free bile acid	2.4	7.6	6.9
Ratio dihydroxy/trihydroxy	0.3	0.3	0.7
Taurine/glycine conjugation ratio	5.6	2.3	0.8

[a] Obtained from intact rats. Represents 1-hr collection after construction of a biliary fistula.
[b] Cultured hepatocytes separated by centrifugation after 1 hr of incubation in media 3.
[c] Obtained from media 3 after 1 hr of incubation.

Quantitative determination of bile acid content in hepatocytes isolated from fed rats at zero time and after 60 min of incubation showed a significant increase in bile acid content, which was 55.4 ± 3.1 nmol/mg protein at zero time and increased to 206.3 ± 8.7 nmol/mg protein after 1 hr of incubation. The bile acid secreted was 1377 ± 20.7 nmol/mg protein after 1 hr. Quantitative analysis of different fractions of bile acids shows that there is a significant difference in the percentage distribution of individual bile acids in BB, HB, and HSB (**Table 5**). The taurine/glycine (T/G) ratio is 5.61 for BB, 2.30 for HB, and 0.84 for HSB after 1 hr of incubation. The percentage of distribution of individual bile acids in HSB was the same after different intervals of incubation time up to 3 hr. The ratio of dihydroxy to trihydroxy bile acids was higher in HSB than in BB. However, the differences in bile acid patterns and relative proportions of primary bile acids in BB and HSB are further evidence that the HSB was synthesized de novo and was not secreted from pre-existing bile acids.

The differences in bile acid distribution in BB and HSB may be due to alterations in physiologic conditions brought on by the large surface area exposed to the incubating medium, the presence of substrates in the medium stimulating specific pathways, ultrastructural cellular changes due to the method of cell

isolation, or differences in the pools of available substrates in the intact animal. The higher ratio of T/G in BB compared with HSB can be the result of a shortage of taurine or the availability of larger amounts of glycine for conjugation in the cultured cells. A shortage of taurine may be attributed to the presence of inadequate amounts of precursor or cofactors needed for its synthesis, and has been identified as a limiting factor in intact humans and rats (5, 11).

It is worthwhile mentioning that the data presented in this part of our work are strong evidence of the integrity of the isolated hepatocytes and that the secreted bile acids are newly synthesized. The hepatocytes have retained the mitochondrial enzymes required for assimilation of respiratory substrates as well as the endoplasmic and microsomal enzymes required for lipid and bile acid synthesis. In addition, there was a fourfold increase in the bile acid content of hepatocytes after 1 hr of incubation and a level of bile acids in the media that could not be explained by secretion alone but indicates active synthesis. The noticeable effect of changing the physiological conditions on bile acid secretion and the clear evidence of a feedback mechanism for bile secretion by hepatocytes may contribute to the support that the bile acids secreted by hepatocytes are newly synthesized and not exuded from the pool content.

In unpublished studies in our laboratory, in intact rats we measured a total output of 180 μmol of bile acids/g liver per min in fasted animals. Our current studies show secretion of bile acids at a rate of 190 μmol/g of isolated hepatocytes per min under carefully controlled conditions. This system may be suitable for further studies on the metabolism of bile acid secretion at a cellular level.

SUMMARY

Using collagenase-treated perfused rat liver, a large number of viable hepatocytes were obtained for culture. The yield was 20–25 × 10^6 cells/g liver with 95% viability. Using different culture media, it was established that the highest yields of bile acids were secreted when some substrates and cofactors were added. Omission of succinate as a respiratory substrate is detrimental to bile acid synthesis. The feedback mechanism of bile acid synthesis is clearly demonstrable in isolated hepatocytes. Different primary bile acids show different inhibitory potencies to bile acid synthesis in hepatocytes.

These experiments describe a model that may be useful in the investigation of liver cell function particularly related to bile acid secretion.

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