26-Hydroxylation of 5β-cholestane-3α,7α,12α-triol by isolated nonparenchymal cells and hepatocytes from rat liver

Svein Dueland, Jan I. Pedersen,[1] Christian A. Drevon, and Ingemar Björkhem

Institute for Nutrition Research, School of Medicine, and Department of Pharmacology, Institute of Pharmacy, University of Oslo, Oslo 3, Norway, and Department of Clinical Chemistry and Research Centre at Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden

Abstract The ability of isolated nonparenchymal and parenchymal rat liver cells to metabolize 5β-cholestane-3α,7α,12α-triol and other bile acid intermediates has been investigated. Incubation of nonparenchymal cells with 5β-cholestane-3α,7α,12α-triol resulted in the formation of one more polar product identified as 5β-cholestane-3α,7α,12α,26-tetrol. The formation was linear with time up to 2 hr and with the number of cells, and showed saturation kinetics with respect to substrate concentration. The maximum rate of conversion was 90 pmol/10^6 cells per hr. Incubation of hepatocytes with the triol resulted in the formation of several more polar products. In addition to 5β-cholestane-3α,7α,12α,26-tetrol, products with retention time on high pressure liquid chromatography identical with 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid and cholic acid were observed. The identity of these products was verified by combined gas–liquid chromatography–mass spectrometry. The rate of conversion was linear with time for about 10 min and saturation with respect to substrate concentration was not attained. At identical substrate concentrations, the total rate of conversion (12.5 nmol/10^6 cells per hr) was at least two orders of magnitude higher than with the nonparenchymal cells. Similar differences in rates of conversion were observed with other C_{27}-bile acid intermediates. It is concluded that the nonparenchymal cells do not play any significant role in the conversion of bile acid intermediates, either under physiological conditions or under experimental conditions where such steroids have been administered intravenously.—**Dueland, S., J. I. Pedersen, C. A. Drevon, and I. Björkhem.** 26-Hydroxylation of 5β-cholestane-3α,7α,12α-triol by isolated nonparenchymal cells and hepatocytes from rat liver. *J. Lipid Res.* 1982. **23:** 1321–1327.

Supplementary key words bile acids • bile acid intermediates • high pressure liquid chromatography

The dominant catabolic pathway of cholesterol in the animal organism involves the conversion of the sterol to primary bile acids, mainly cholic and chenodeoxycholic acid. Our present knowledge of the nature and the sequence of reactions of this conversion is based both on in vitro and in vivo studies (1, 2). A main experimental approach has been to administer radioactive synthetic hypothetical intermediates to either rats (for review see ref. 1) or patients (3–7) with bile fistula (or biliary diversion), and to analyze the products secreted in the bile. The assumption has been made that these exogenously administered intermediates are taken up by the liver cells and handled in a way similar to the endogenously formed metabolites.

Recently it has been shown that both the hepatocytes and the nonparenchymal cells of the liver are able to take up lipids and steroids such as cholesterol (8–10) and vitamin D_3 (11) from blood in vivo. It can therefore not be excluded that both types of liver cells may be able to metabolize intravenously administered bile acid intermediates.

In the present work we have studied the ability of isolated nonparenchymal liver cells from rats to hydroxylate 5β-cholestane-3α,7α,12α-triol and other C_{27}-steroids. It is shown that these cells have the capacity to 26-hydroxylate such intermediates but at rates at least two orders of magnitude lower than isolated hepatocytes.

MATERIALS AND METHODS

Chemicals

Pronase (B-grade) was purchased from Calbiochem AG, Lucerne, Switzerland, and collagenase (Type I) was

Abbreviations and nomenclature: GLC–MS, gas–liquid chromatography–mass spectrometry; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid; HPLC, high pressure liquid chromatography; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. 26-Hydroxylation, this hydroxylation may be a 26-hydroxylation or a 27-hydroxylation (at the 25-pro-R-methyl group or the 25-pro-S-methyl group, respectively). For a more detailed discussion, see footnote in ref. 16.

[1] To whom correspondence should be sent at Institute for Nutrition Research, University of Oslo, P.O. Box 1046, Blindern, Oslo 3, Norway.

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obtained from Sigma Chemical Co., St. Louis, MO. 5-Cholestene-3β,7α-diol, 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,26-tetrol, and the 25*R*-isomer of 3α,7α,12α-trihydroxy-5β-cholestanoic acid were synthesized and purified as described previously (12–16). [7β-^{3}H]5-Cholestene-3β,7α-diol (6 Ci/mol), [7β-^{3}H]5β-cholestane-3α,7α-diol (7 Ci/mol), [7β-^{3}H]5β-cholestane-3α,7α,12α-triol (7 Ci/mol), [6β-^{3}H]7α-hydroxy-4-cholesten-3-one (7 Ci/mol), and [7β-^{3}H]5β-cholestane-3α,7α,12α,26-tetrol (7 Ci/mol) were prepared as previously described (16–18). The radioactive steroids were purified by HPLC prior to use (15). [*Carboxyl*-^{14}C]cholic acid used as a reference substance was from the Radiochemical Centre, Amersham, England. Other chemicals and solvents were standard commercial high purity materials.

Animals

Male Wistar rats (about 250 g body weight) were fed ordinary lab chow. The animals were given water and food ad libitum until the liver perfusion started at about 9 AM.

Preparation of liver cells

Isolated liver cells were obtained by a modification (19) of the method of Berry and Friend (20). In short, the liver was removed from the animal and perfused in vitro, first with a calcium-free buffer for 5–10 min, followed with a buffer containing 4 mM $CaCl_2$ and 0.05% (w/v) collagenase. The perfusion rate was about 30 ml/min and the cells were separated after about 10 min. The hepatocytes were separated from the nonparenchymal cells by differential centrifugation (21) in a medium containing 1% bovine serum albumin (fatty acid-free). The nonparenchymal cells were separated from the parenchymal cells by use of the pronase method (22). In this case, the partly purified nonparenchymal cell suspension (<1% contaminated by parenchymal cells) was incubated with 0.25% (w/v) pronase for 90 min on a shaking water bath (80 oscillations/min) at 37°C. Pronase will selectively destroy all the hepatocytes while nonparenchymal cells remain intact (22). The isolated cells were finally resuspended in the incubation buffer at a concentration of approximately 5 $\times 10^6$ cells/ml. No cross contamination was observed in the two cell populations as evaluated by microscopy. The viability of the isolated cells was estimated by the trypan blue (0.04%) exclusion test (23). The viability of the cells was higher than 95% in all preparations.

Incubation, extraction, and chromatographic procedures

The isolated cells suspended in 2 ml of buffer were incubated in 50-ml Erlenmeyer flasks on a shaking water bath (75 oscillations/min) at 37°C for varying periods of time. The incubation buffer contained 0.146 M NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 2 mM $CaCl_2$, 0.7 mM Na_2HPO_4/KH_2PO_4 buffer (pH 7.5), 20 mM Hepes buffer (pH 7.5), and 10 mg/ml bovine serum albumin. The osmolarity was approximately 300 mosm/l. The reaction was started by the addition of 25 nmol of 5β-cholestane-3α,7α,12α-triol containing approximately 250,000 cpm in 20 μl or less of ethanol. Further details are given in the legends to the table and figures.

The reaction was terminated by the addition of 135 μl of 1 N HCl and the reaction mixtures were immediately extracted with ethyl acetate (24). The solvent was evaporated under a stream of N_2, and the residue was redissolved in 100 μl of methanol. Aliquots of the extracts (usually 40 μl) were subsequently analyzed by HPLC using a Zorbax ODS column (4.6 $\times$ 250 mm) and 7.5% water in methanol as eluting solvent (1 ml/min). One-ml fractions were collected and, after addition of 5 ml of counting solution (Dilusolve, Packard Instrument), they were counted in a Tri-Carb Liquid Scintillation Spectrometer at about 50% efficiency. When necessary, quenching was corrected for by use of tritium-labeled cholesterol as external standard. The amount of product formed during the incubation was calculated from the percentage distribution of radioactivity. In incubations with hepatocytes, the calculations had to be corrected for the radioactivity that remained in the water phase after extraction with ethyl acetate (5–20%). The recovery of radioactivity from the column was essentially complete. Tritium-labeled bile acid intermediates were used as external standards to identify the products. For final identification the peak fractions of the products were collected. The solvent was evaporated and, after conversion to the trimethylsilyl derivative (25), the material was analyzed by combined gas–liquid chromatography–mass spectrometry (16).

All experimental series were repeated two or three times.

RESULTS

Metabolism of 5β-cholestane-3α,7α,12α-triol by nonparenchymal cells

After incubation of nonparenchymal cells with the triol, the combined cells and medium were extracted.

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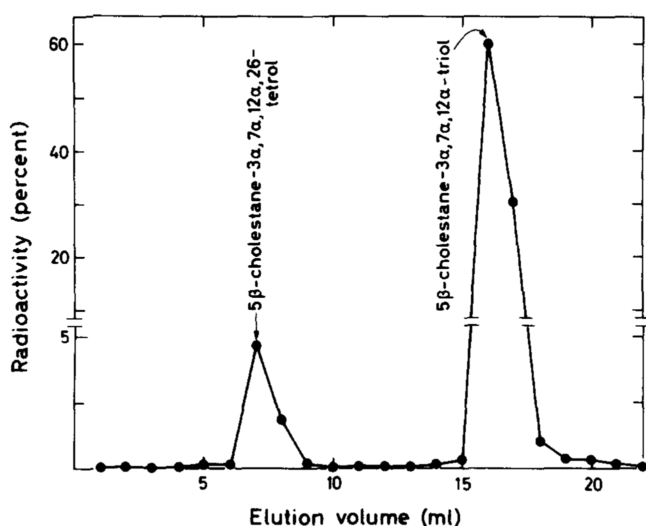

Fig. 1. Reversed phase HPLC of an ethyl acetate extract of nonparenchymal cells after incubation with 5β-cholestane-3α,7α,12α-triol. Nonparenchymal cells, 8.8 × 10^6, were incubated for 2 hr, extracted, and analyzed by HPLC as described in Materials and Methods.

High pressure liquid chromatography of these extracts revealed formation of one more polar product (**Fig. 1**). The retention time of the product was identical to that of ^{3}H-labeled 5β-cholestane-3α,7α,12α,26-tetrol. No peaks corresponding to more polar products were observed in the chromatogram (Fig. 1) even after prolonged incubation with only labeled substrate added. The trimethylsilyl derivative of the product was analyzed by GLC–MS by monitoring the ion at m/e 544 (16). Only one peak with a retention time identical to authentic 5β-cholestane-3α,7α,12α,26-tetrol was observed.

The rate of formation of 5β-cholestane-3α,7α,12α,26-tetrol was linear with time up to at least 2 hr of incubation (**Fig. 2A**). The rate was also strictly linear with the number of nonparenchymal cells up to 10^7 cells per 2 ml of incubation volume (Fig. 2B). The rate of product formation increased with substrate concentration up to about 6 μmol/l when a saturation level was reached (Fig. 2C). The maximum rate of formation of 5β-cholestane-3α,7α,12α,26-tetrol was about 90 pmol per 10^6 cells per hr.

Conversion of 5β-cholestane-3α,7α,12α-triol by isolated hepatocytes

When the ethyl acetate extracts of incubations of hepatocytes with 5β-cholestane-3α,7α,12α-triol were analyzed by HPLC, the chromatograms differed from that described above. In addition to a peak with retention time identical to 5β-cholestane-3α,7α,12α,26-tetrol, peaks with retention time corresponding to acidic products (3α,7α,12α-trihydroxy-5β-cholestanoic acid and cholic acid) appeared (**Fig. 3**). The trimethylsilyl ether of the product 5β-cholestane-3α,7α,12α,26-tetrol was analyzed by GLC–MS by monitoring the ions at m/e 253, m/e 544, and m/e 634 (cf. ref. 16). A major peak with a retention time identical to authentic 5β-

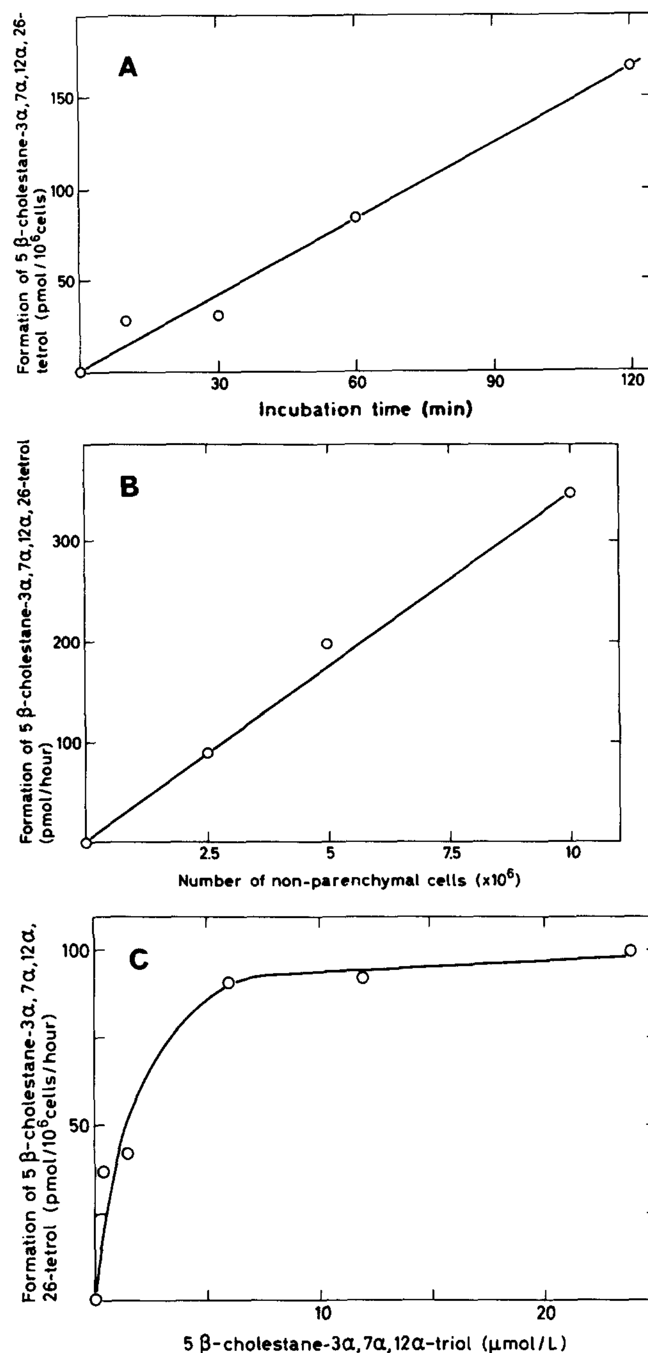

Fig. 2. Effect of time (A), number of cells (B), and substrate concentration (C) on the rate of conversion of 5β-cholestane-3α,7α,12α-triol by nonparenchymal liver cells. Except for the variations as shown, standard incubation conditions were used (see Materials and Methods). The number of cells was 8.8 × 10^6 in A and 6 × 10^6 in C. The time of incubation was 1 hr in B and C.

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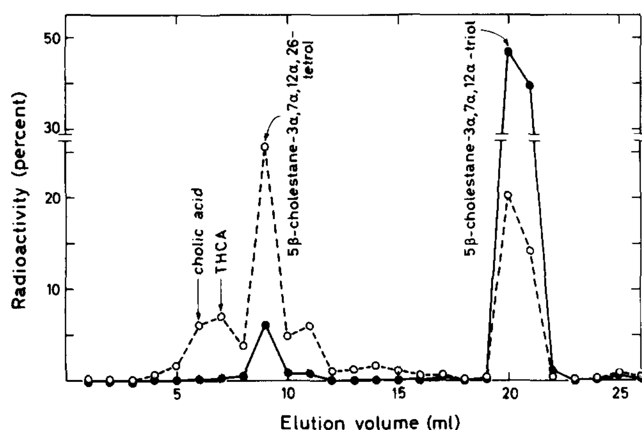

Fig. 3. Reversed phase HPLC of ethyl acetate extracts of hepatocytes after incubation with 5β-cholestane-3α,7α,12α-triol. Hepatocytes, 2.5 × 10^6, were incubated, extracted, and analyzed by HPLC as described in Materials and Methods. The diagrams show the distribution of radioactivity after 5 min (● —— ●) and 60 min (O - - - O) of incubation. The arrows indicate the elution volumes of the respective standards; THCA = 3α,7α,12α-trihydroxy-5β-cholestanoic acid. (A precolumn, RP-18, was connected in front of the Zorbax ODS column; this explains the differences in elution volumes compared to those in Fig. 1.)

cholestane-3α,7α,12α,26-tetrol was observed in each tracing. In addition, a small peak (corresponding to less than 10%) was obtained with a retention time identical to 5β-cholestane-3α,7α,12α,25-tetrol (cf. ref. 16). The identity of the latter compound was further established by demonstration of the presence of the ion at m/e 131, which is specific for a trimethylsilyl group at C-25 (15). The materials corresponding to 3α,7α,12α-trihydroxy-5β-cholestanoic acid and cholic acid were converted into methyl ester trimethylsilyl derivative and then analyzed by GLC–MS. The identity of the 3α,7α,12α-trihydroxy-5β-cholestanoic acid was confirmed by tracing of the ions at m/e 500 and m/e 410 (cf. ref. 16), and the identity of cholic acid was confirmed by tracing of the ions at m/e 368, m/e 623, and m/e 638 (cf. ref. 26). In each tracing a major peak was obtained with a retention time corresponding to authentic compound. The rate of formation of the product corresponding to 5β-cholestane-3α,7α,12α,26-tetrol was linear only for about 15 min of incubation (**Fig. 4**). The most polar products, cholic acid and 3α,7α,12α-trihydroxy-5β-cholestanoic acid increased linearly with time up to one hour (Fig. 4) when most of the substrate had been transformed.

The total product formation was linear with time only during the first 10–15 min of incubation (**Fig. 5A**). The total product formation was linear with the number of hepatocytes up to at least 10^7 cells per 2 ml of incubation volume (Fig. 5B). The rate of product formation increased with the substrate concentration, but without showing saturation kinetics. At a substrate concentration of 12 μM the initial rate of substrate conversion was about 12.5 nmol per 10^6 cells per hr, i.e., about two orders of magnitude higher than found with nonparenchymal cells at the same substrate concentration (Fig. 5C).

Conversion of other C_{27}-steroids by isolated hepatocytes and nonparenchymal cells

A number of other bile acid intermediates were tested as substrates for the conversion by the two cell types. The results presented in **Table 1** show that the nonparenchymal cells are able to transform all C_{27}-steroids tested into more polar products, but at rates about two orders of magnitude lower than the hepatocytes. With all substrates only one product peak was observed when incubated with nonparenchymal cells. The retention time on HPLC was identical to the corresponding 26-hydroxylated derivative (cf. ref. 15). When incubated with the hepatocytes, a more complex pattern of several polar products was observed. A more detailed study of this pattern is under way.

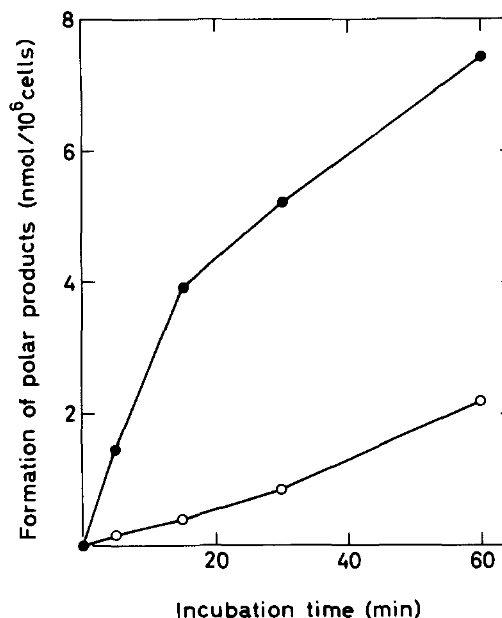

Fig. 4. Effect of time on the distribution of radioactivity within polar products from incubations of hepatocytes with 5β-cholestane-3α,7α,12α-triol. Hepatocytes, 2.5 × 10^6, were incubated under standard conditions (see Materials and Methods) for varying periods of time. The extracts were analyzed by HPLC as in Fig. 3. The relative amounts of product corresponding to 5β-cholestane-3α,7α,12α,26-tetrol, ● —— ●, and to the sum of cholic acid and trihydroxy-5β-cholestanoic acid, O —— O, are plotted as a function of time. (The amount of products in the aqueous phase that increased from about 10% of total activity at 5 min to about 17% at 60 min was taken into account when the conversion was calculated.)

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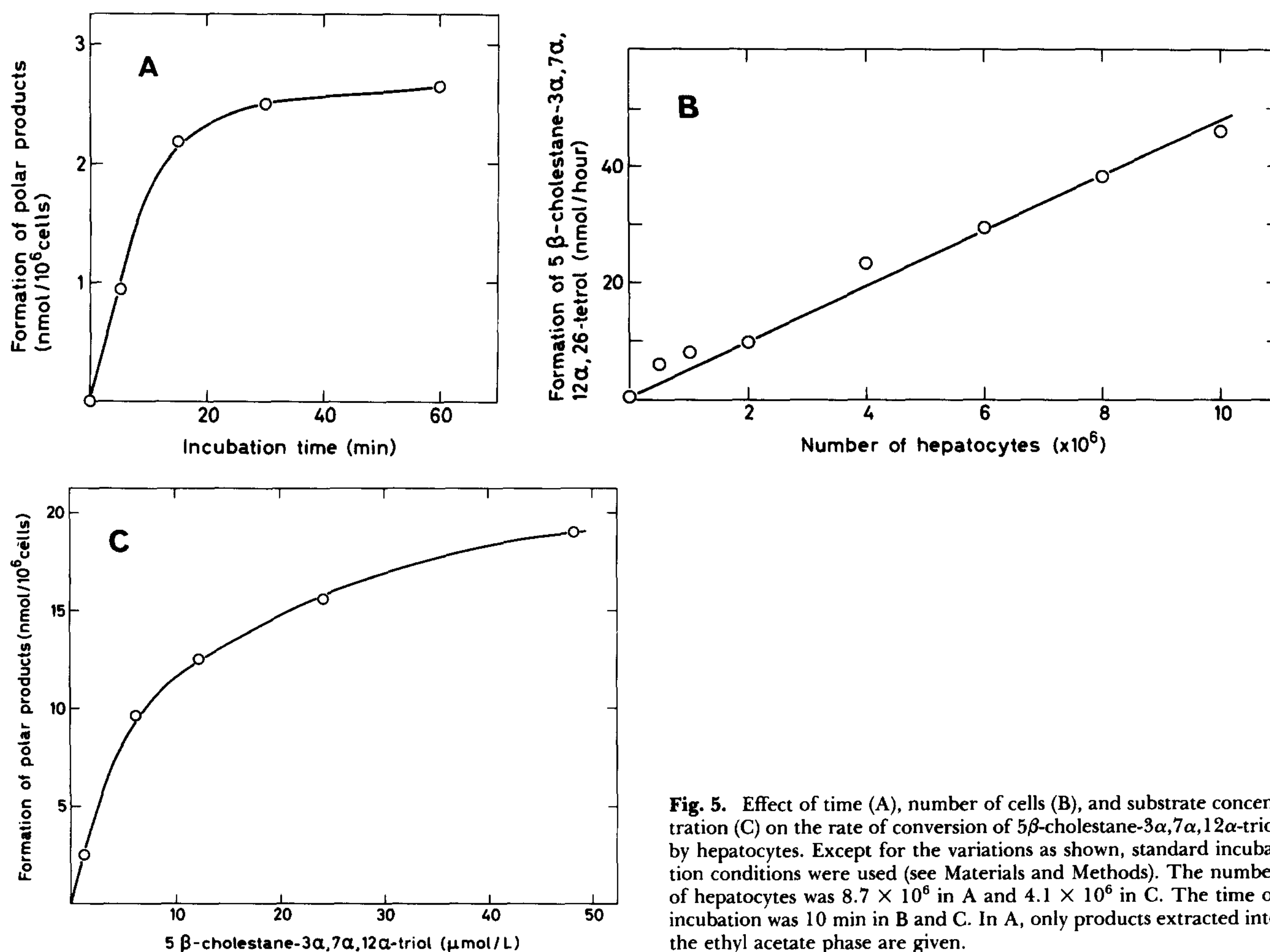

Fig. 5. Effect of time (A), number of cells (B), and substrate concentration (C) on the rate of conversion of 5β-cholestane-3α,7α,12α-triol by hepatocytes. Except for the variations as shown, standard incubation conditions were used (see Materials and Methods). The number of hepatocytes was 8.7×10^6 in A and 4.1×10^6 in C. The time of incubation was 10 min in B and C. In A, only products extracted into the ethyl acetate phase are given.

DISCUSSION

Previous studies have shown that isolated rat liver cells have the capacity to synthesize and secrete bile acids (27–30). No attempts were made to separate the nonparenchymal cells from the hepatocytes in these previous studies.

In this work we have shown that isolated hepatocytes are able to convert 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α,26-tetrol and more polar products at high rates. It was somewhat more unexpected that the nonparenchymal cells also had the capacity to transform the triol into 5β-cholestane-3α,7α,12α,26-tetrol. The 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol by the nonparenchymal cells could not be explained by contamination of this cell population with hepatocytes. We treated the liver cells with pronase in order to obtain a pure preparation of nonparenchymal cells. This procedure effectively destroys all hepatocytes and leaves only the nonparenchymal cells intact (22). By light microscopy we confirmed the absence of contaminating parenchymal cells. Also, the different pattern of product formation makes it less likely that the 26-hydroxylase activity of the nonparenchymal cells is due to contaminating hepatocytes. Thus, saturation kinetics with respect to substrate was

TABLE 1. Conversion of bile acid intermediates by hepatocytes and nonparenchymal cells

Substrate	Hepatocytes	Nonparenchymal Cells
	nmol / 10⁶ cells · hr	
5-Cholestene-3β,7α-diol	2.5	0.07–0.10
7α-Hydroxy-4-cholesten-3-one	4.7	0.04
5β-Cholestane-3α,7α-diol	11.4	0.05–0.07
5β-Cholestane-3α,7α,12α-triol	14.7	0.05–0.09

The incubation, extraction, and chromatographic procedures were as described in Materials and Methods. The time of incubation was 10 min with the hepatocytes and 1 hr with the nonparenchymal cells. The results represent total conversion expressed per hr into several polar products in incubations with hepatocytes and into one (26-) hydroxylated product in incubations with nonparenchymal cells.

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observed with the nonparenchymal cells, which was not the case with hepatocytes.

Furthermore, 5β-cholestane-3α,7α,12α,26-tetrol was the only metabolite formed by the nonparenchymal cells. No more polar products were observed even up to 2 hr of incubation. In contrast, when hepatocytes were incubated, more polar products appeared in increasing amounts after about 5 min of incubation, while the amount of 5β-cholestane-3α,7α,12α,26-tetrol leveled off after 15 min incubation time.

The maximum rate of hydroxylation of 5β-cholestane-3α,7α,12α-triol by nonparenchymal cells was only about 1/100 of that observed with hepatocytes calculated per 10^6 cells. About 70% of the cells of the liver consist of parenchymal cells (31). It therefore appears unlikely that the nonparenchymal cells can play any significant role in the metabolism of bile acid intermediates. This probably also holds true under experimental conditions in vivo where bile acid intermediates have been exogenously administered. Preliminary experiments indicate that the uptake of a trace amount of 5β-cholestane-3α,7α,12α-triol from the circulation by the nonparenchymal cells is rather small. Thirty min after intravenous administration of ^{3}H-labeled 5β-cholestane-3α,7α,12α-triol to the rat, less than 5% of the activity recovered in the isolated liver cells was accounted for in the nonparenchymal cells, the remainder in the hepatocytes (unpublished results). Even if the nonparenchymal cells have a certain capacity to take up such intermediates from the circulation, their metabolism most likely takes place primarily after transfer to the hepatocytes.

The very active metabolism of 5β-cholestane-3α,7α,12α-triol observed with isolated parenchymal liver cells demonstrates that this is a suitable in vitro system to study the reactions involved in bile acid formation in mammals.

The skillful technical assistance of Eva Torma Grabner is appreciated. This work was supported by the Norwegian Research Council for Science and the Humanities and by the Swedish Medical Research Council (project 03X-3141).

Manuscript received 4 January 1982 and in revised form 2 June 1982.

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