Fig. 2 is an example of the a.c. polarograms obtained with the set-up described. The corresponding d.c. polarogram is shown in Fig. 2a.

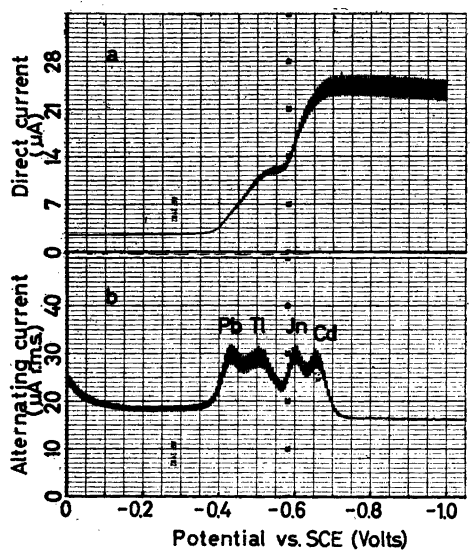

Fig. 2. a) D.c. polarogram of a mixture of 0.3 mM Pb^{2+}, 1.0 mM Tl^{+}, 0.5 mM In^{3+}, 0.5 mM Cd^{2+}, and 0.1 M HCl in the absence of air oxygen; no extra maximum suppressor added; *b)* a.c. polarogram of the same electrolyte solution but measured in the presence of air oxygen; a.c. frequency 160 Hz; a.c. amplitude 5 mV r.m.s. Damping grade "4" on the recorder "PO4" in both cases.

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1. Miller, D. M. *Can. J. Chem.* **34** (1956) 942; **35** (1957) 1589.
2. Head, W. F. *Anal. Chim. Acta* **23** (1960) 297.
3. Beckman, H. and Gauer, W. O. *Anal. Chem.* **38** (1966) 1434.
4. Breyer, B. and Bauer, H. H. *Alternating Current Polarography and Tensammetry*, Interscience, New York 1963.

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On the Role of Δ^4-3α-Hydroxysterols in the Biosynthesis of Bile Acids

Bile Acids and Steroids 191*

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In the biosynthesis of bile acids the Δ^5-3β-hydroxy configuration of cholesterol is transformed into the 3α-hydroxy-5β configuration of chenodeoxycholic acid and cholic acid by means of the intermediary formation of Δ^4-3-ketosteroids.[2] Evidence has been presented that the Δ^4-3-ketosteroids are 7α-hydroxycholest-4-en-3-one and 7α,12α-dihydroxycholest-4-en-3-one.[3,4] Studies on the metabolism of these compounds *in vitro* indicate that the major pathway for their further metabolism involves the conversion to the corresponding 3-keto-5β-steroids followed by reduction of the 3-keto group to yield 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol.[3-6] However, it has been suggested that in the biosynthesis of 5β-cholestane-3α,7α,12α-triol, 7α-hydroxycholest-4-en-3-one is converted to 5β-cholestane-3α,7α-diol also by means of the intermediate formation of cholest-4-ene-3α,7α-diol.[3] Hutton and Boyd [7] have reported the formation of cholest-4-ene-3α,7α-diol from 7α-hydroxycholest-4-en-3-one in the presence of a partially purified supernatant fraction from rat liver homogenate. Cholest-4-ene-3α,7α-diol was, however, a minor metabolite and the major pathway for the metabolism of 7α-hydroxycholest-4-en-3-one under these conditions involved the conversion into 7α-hydroxy-5β-cholestan-3-one and subsequently 5β-cholestane-3α,7α-diol. Similar findings were made by Björkhem and Danielsson [8] in a study of the formation and metabolism of various Δ^4-cholestenols including cholest-4-ene-3α,7α-diol. The results indicated that the major pathway for the metabolism *in vitro* of cholest-4-ene-

* The preceding communication in this series is Ref. 1. The following systematic names are given to compounds referred to by trivial names: chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid.

3α,7α-diol and cholest-4-ene-3α,7α,12α-triol was the oxidation to the corresponding Δ^4-3-ketosteroids. In an earlier investigation [9] it was shown that 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol was converted to cholic acid in the bile fistula rat with complete loss of tritium demonstrating the intermediary formation of a 3-keto compound. The experiments did not allow any conclusion regarding the stage at which the oxidation occurred, but taken in conjunction with the experiments *in vitro* referred to above, it would seem probable that cholest-4-ene-3α,7α,12α-triol is converted to cholic acid by means of the intermediary formation of 7α,12α-dihydroxycholest-4-en-3-one. However, it can be argued that *in vivo* cholest-4-ene-3α,7α-diol and cholest-4-ene-3α,7α,12α-triol can be transformed into 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol by direct saturation of the double bond and that the observed loss of tritium from 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol during its conversion to cholic acid could be due to the existence of a rapid equilibrium between 5β-cholestane-3α,7α,12α-triol and 7α,12α-dihydroxy-5β-cholestan-3-one or between another 3α-hydroxy-5β compound and a 3-keto-5β compound in a later stage of the pathway for cholic acid formation.

To provide further information on the role of cholest-4-ene-3α,7α-diol and cholest-4-ene-3α,7α,12α-triol as intermediates in the conversion of cholesterol to bile acids, the metabolism of 3β-^{3}H-labeled cholest-4-ene-3α,7α-diol, 5β-cholestane-3α,7α-diol, and 5β-cholestane-3α,7α,12α-triol has been studied in the bile fistula rat.

Materials. 4-^{14}C-7α-Hydroxycholest-4-en-3-one (m.p. 177°, reported [10] m.p. 183—184°; specific radioactivity 1 μC/mg) was prepared from 4-^{14}C-cholesterol (Radiochemical Centre, Amersham, England) according to the procedure of Naqvi and Boyd as reported by Hutton and Boyd.[7] 4-^{14}C-7α-Hydroxycholest-4-en-3-one, 9 mg, was reduced with sodium borohydride in 70 % aqueous methanol and cholest-4-ene-3α,7α-diol was isolated from the reduction mixture as described previously.[8] The 4-^{14}C-cholest-4-ene-3α,7α-diol obtained, altogether 0.4 μC, gave a single spot in various thin layer chromatographic systems and the identity was further established by crystallizing part of the material together with authentic, unlabeled cholest-4-ene-3α,7α-diol. The specific radioactivity remained constant through several recrystallizations. 3β-^{3}H-Cholest-4-ene-3α,7α-diol was prepared by reduction of 10 mg of 7α-hydroxycholest-4-en-3-one, m.p. 183—184°, with 5 mg of tritium-labeled sodium borohydride (Radiochemical Centre) in 70 % aqueous methanol. The product was isolated and identified as described previously [8] and above. The specific radioactivity of the 3β-^{3}H-cholest-4-ene-3α,7α-diol was estimated to be 8 μC/mg.

4-^{14}C-5β-Cholestane-3α,7α-diol (m.p. 80—84°, reported [11] m.p. 84—86°; specific radioactivity 0.04 μC/mg) was prepared by electrolytic coupling of 4-^{14}C-chenodeoxycholic acid and isovaleric acid as described by Bergström and Krabisch.[11] 4-^{14}C-Chenodeoxycholic acid was prepared by biosynthesis from 4-^{14}C-cholesterol in a bile fistula rat treated with injections of triiodo-L-thyronine (200 μg/kg body weight/day) as described by Strand.[12] 4-^{14}C-Chenodeoxycholic acid was isolated by chromatography on phase system F1 [13] of hydrolyzed bile collected for one week after administration of 4-^{14}C-cholesterol. The total yield of 4-^{14}C-chenodeoxycholic acid was about 100 mg. 3β-^{3}H-5β-Cholestane-3α,7α-diol (m.p. 81—83°; specific radioactivity 3 μC/mg) was prepared by reduction of 10 mg of 7α-hydroxy-5β-cholestan-3-one (m.p. 120—121°, reported [14] m.p. 121—122°) with 5 mg of tritium-labeled sodium borohydride in methanol. 3β-^{3}H-5β-Cholestane-3α,7α-diol was isolated from the reduction mixture by preparative thin layer chromatography using Kieselgel G (Merck, Darmstadt, Germany) as adsorbent and benzene-ethyl acetate, 3:7, as solvent.

4-^{14}C-5β-Cholestane-3α,7α,12α-triol (m.p. 186—188°, reported [11] m.p. 184—185°; specific radioactivity 0.04 μC/mg) was prepared as described recently.[9] 3β-^{3}H-5β-Cholestane-3α,7α,12α-triol (m.p. 184°; specific radioactivity 3 μC/mg) was prepared by reduction of 10 mg of 7α,12α-dihydroxy-5β-cholestan-3-one (m.p. 208—210°, reported [5] m.p. 208—209°) with 5 mg of tritium-labeled sodium borohydride in methanol and was isolated by means of preparative thin layer chromatography with ethyl acetate as solvent.

Animal experiments. Male rats of the Sprague Dawley strain weighing about 200 g were used. Bile fistulas were prepared in the usual manner. The labeled compounds were administered intraperitoneally in an emulsion stabilized with bovine serum albumin. Bile was collected in 24-hour portions. Hydrolysis of bile was carried out with 1 M sodium hydroxide in 50 % aqueous ethanol in sealed steel tubes at 110° for 12 h. The hydrolyzed bile was acidified with hydrochloric acid and extracted with ether. The residue of the ether extract was chromatographed on hydrophobic Hyflo Super-Cel with phase systeme C1.[13] The cholic acid

Table 1. Formation of cholic acid from 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α-diol, 3β-^{3}H-4-^{14}C-5β-cholestane-3α,7α-diol and 3β-^{3}H-4-^{14}C-5β-cholestane-3α,7α,12α-triol.

Compound	^{3}H cpm	^{14}C cpm	^{3}H/^{14}C	Per cent ^{3}H retained
Administered 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α-diol	22 902	6252	3.7	100
Cholic acid isolated from rat 1	1 220	5732	0.21	6
Cholic acid isolated from rat 2	559	3306	0.17	5
Administered 3β-^{3}H-4-^{14}C-5β-cholestane-3α,7α-diol	20 393	1537	13.5	100
Cholic acid isolated from rat 3	10 959	1053	10.4	77
Cholic acid isolated from rat 4	2 418	224	10.8	80
Administered 3β-^{3}H-4-^{14}C-5β-cholestane-3α,7α,12α-triol	26 768	1517	17.7	100
Cholic acid isolated from rat 5	27 579	2086	13.2	75
Cholic acid isolated from rat 6	9 946	650	15.3	86

was isolated and was crystallized to constant specific radioactivity before determination of isotope content.

Radioactivity assay. Simultaneous determination of ^{3}H and ^{14}C was performed in a Packard Tri-Carb spectrometer, model 4322. The cholic acid was converted into methyl ester with diazomethane before addition of the scintillating solution (4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene in 1000 ml of toluene). The discriminator-ratio method was used and internal standards of ^{3}H and ^{14}C were used to correct for quenching.

Results and discussion. Table 1 summarizes the determinations of ^{3}H/^{14}C ratios in the cholic acid samples isolated from bile of bile fistula rats treated with injection of 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α-diol (1 mg), 3β-^{3}H-4-^{14}C-5β-cholestane-3α,7α-diol (1 mg), or 3β-^{3}H-4-^{14}C-5β-cholestane-3α,7α,12α-triol (1 mg). Whereas more than 90 % of the tritium label in 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α-diol was lost during its conversion to cholic acid, 3β-^{3}H-4-^{14}C-5β-cholestane-3α,7α-diol as well as 3β-^{3}H-4-^{14}C-5β-cholestane-3α,7α,12α-triol were converted to cholic acid with retention of more than 75 % of the tritium label. As reported previously,[9] 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol is converted to cholic acid with loss of more than 90 % of the tritium label. Since the main part of the tritium label in 3β-^{3}H-labeled 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol is retained during cholic acid formation, it can be concluded that the oxidation resulting in the loss of tritium label from 3β-^{3}H-labeled cholest-4-ene-3α,7α-diol and cholest-4-ene-3α,7α.12α-triol occurs prior to saturation of the double bond. Thus, the Δ^{4}-cholestenols are converted to cholic acid predominantly be means of the intermediary formation of Δ^{4}-3-ketosteroids. The present experiments do not provide *per se* any information concerning the role of Δ^{4}-cholestenols in bile acid biosynthesis. However, when the present results are taken in conjunction with those of a previous investigation on the formation and metabolism of Δ^{4}-cholestenols *in vitro*,[8] it appears that cholest-4-ene-3α,7α-diol and cholest-4-ene-3α,7α,12α-triol are not important intermediates in the conversion of cholesterol to bile acids.

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1. Berséus, O. and Björkhem, I. *European J. Biochem.* **2** (1967) 503.
2. Gréen, K. and Samuelsson, B. *J. Biol. Chem.* **239** (1964) 2804.
3. Mendelsohn, D., Mendelsohn, L. and Staple, E. *Biochemistry* **5** (1966) 1286.
4. Danielsson, H. and Einarsson, K. *J. Biol. Chem.* **241** (1966) 1449.
5. Berséus, O., Danielsson, H. and Kallner, A. *J. Biol. Chem.* **240** (1965) 2396.
6. Berséus, O. *European J. Biochem.* **2** (1967) 493.
7. Boyd, G. S. and Hutton, H. R. B. *Biochim. Biophys. Acta* **116** (1966) 336, 362.
8. Björkhem, I. and Danielsson, H. *European J. Biochem.* **2** (1967) 403.
9. Björkhem, I. and Danielsson, H. *Acta Chem. Scand.* **19** (1965) 2298.
10. Danielsson, H. *Acta Chem. Scand.* **15** (1961) 242.
11. Bergström, S. and Krabisch, L. *Acta Chem. Scand.* **11** (1957) 1067.
12. Strand, O. *J. Lipid Res.* **4** (1963) 305.
13. Norman, A. and Sjövall, J. *J. Biol. Chem.* **233** (1958) 872.
14. Yamasaki, K., Kawahara, T. and Shimizu, K. *Yonaga Acta Med.* **4** (1959) 37.

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Adsorption Effects in Gel Filtration of Humic Acid

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The remarkable success of the gel filtration method in the study of a number of complex systems has caused its application also to the investigation of the ill-defined organic matter in soil which is usually called humus or humic acids.[1-16] This presumably polyaromatic and certainly polyelectrolytic matter offers special difficulties in the evaluation of the gel filtration results. These difficulties are not always fully realized by those who use the method.

All aromatic compounds are to some extent adsorbed by Sephadex gels and the polyaromatic nature of the humic acids leads to serious adsorption effects. Posner [12] has correctly pointed out that adsorption takes place in salt medium but his conclusions about a suitable elution procedure have not been corroborated by the present study.

Experimental. A great number of humic acid samples of different origin and obtained by different extraction methods have been studied. The same adsorption effects were found in all cases and only two typical examples will be presented here. The sample used was a neutral solution of the most salt-sensitive part of the humic acid obtained from a *chernozem* soil. Sephadex G15 and G100 from Pharmacia have been used for the gel filtrations. The columns had the dimensions 38 × 2.5 cm. The elution velocity was controlled by a peristaltic pump to 24 ml/h. The columns were always equilibrated with the first elution solution before the start of the elution.

The UV absorption at 253.6 nm was registered on an LKB Uvicord instrument. 3 ml of the samples were added to the column.

Results and discussion. We will first treat the case without any other separation than that due to adsorption. This is exemplified by the gel filtrations on G15 with this particular sample. By elution with 0.05 M NaCl (Fig. 1a) only one fraction is obtained even after 24 h of elution or more. A change of eluent to distilled water leads, however, to the development after some hours of a clearly visible fraction which is also registered at the Uvicord diagram. The appearance of this fraction coincides with the disappearance of salt in the eluted solution as found by conductance measurements.

It thus seems as if *the humic acid molecules in the salt solution are strongly adsorbed on the available adsorption sites of the gel* and not until all adsorption sites are occupied the rest of the sample will pass through the column completely excluded. At the subsequent elution with distilled water the humic acid molecules are released from the adsorption sites. They are then accumulated in the front of the solution with low ionic strength and there gradually form a rather sharp fraction.

This interpretation is corroborated by the following experiment. The excluded fraction was concentrated to the original volume and rerun through the column in ecxatly the same way as earlier (Fig. 1b). The same amount was adsorbed and only a small fraction was completely excluded.