Comparative Studies of 'Bile Salts'

10. BILE SALTS OF THE KING PENGUIN, *APTENODITES PATAGONICA**

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The scanty information about the chemical nature of the bile acids of birds has not been previously collected together and this is now done in Table 1. Claims have been made for the detection of free bile acids in bird bile (e.g. Maeda, 1938), but it is believed that these acids generally occur conjugated with taurine. Haslewood & Sjövall (1954) could detect no glycine conjugates in the bile salts of eight species of birds, and Wiggins (1955*a*), in a careful examination of chicken bile by countercurrent distribution methods, failed to find either free bile acids or glycine conjugates.

The present report is of an examination of the bile salts of the King penguin, *Aptenodites patagonica*, and includes remarks on the chemical nature of tetrahydroxynorsterocholanic acid.

RESULTS

By methods previously used in these studies, King penguin bile was shown to contain chenodeoxycholic acid, cholic acid and tetrahydroxynorsterocholanic

* Part 9: Haslewood (1956).

acid. At least four other 'bile acids' may be present in the bile; none of these appeared to be substances known to us. The proportion of cholic and tetrahydroxynorsterocholanic acids, taken together, was much greater than in chicken bile, but their striking chemical similarity prevented complete separation of these acids or their esters and hence any reliable estimation of the amounts of each.

Ethyl cholate and 'ethyl tetrahydroxynorsterocholanic' ran at virtually the same rate on our paper chromatograms. These esters apparently formed mixed crystals; the melting point of one of our preparations could be raised to about 200° by recrystallization from benzene. Material of this purity on hydrolysis yielded a sample of tetrahydroxynorsterocholanic acid, m.p. about 214°, with characteristic behaviour, apparently substantially the same substance as a sample earlier given to us by Professor T. Kazuno (see Discussion). The ethyl esters of our material and that of Kazuno likewise appeared to be identical. Elementary analyses of our product gave figures corresponding to $C_{26}H_{44}O_5$, i.e. to ethyl cholate, rather than to the

Table 1. *Bile acids of birds*

Species	Bile acids isolated	Reference
Domestic chicken	Chenodeoxycholic (3 α :7 α -dihydroxy-cholanic) Isolithocholic Cholic (3 α :7 α :12 α -trihydroxycholanic) Tetrahydroxynorsterocholanic 3 α -Hydroxy-7-oxocholanic 3-Oxochola-4:6-dienic*	Yonemura (1926); Maeda (1938); Takahashi (1938) Hosizima, Takata, Uraki & Sibuya (1930) Yamasaki (1933) Yamasaki (1951) Wiggins (1955 <i>a</i>) Wiggins (1955 <i>b</i>)
Domestic duck (<i>Anas domestica</i>); wigeon, <i>Anas (Mareca) penelope</i> ; turkey (<i>Meleagris gallopavo</i>)	Cholic; chenodeoxycholic	Ishihara & Mori (1938)
Domestic goose	Chenodeoxycholic	Marsson (1849 <i>a, b</i>); Windaus, Bohne & Schwarzkopf (1924)
Pheasant, <i>Phasianus colchicus</i> <i>karpowi</i>	Chenodeoxycholic	Ohta (1939 <i>a</i>)
Owl	Cholic; chenodeoxycholic	Ono & Tamura (1953)
King penguin, <i>Aptenodites</i> <i>patagonica</i>	Cholic; chenodeoxycholic; tetrahydroxynorsterocholanic	Present work

* May be an artifact derived from 7 α -hydroxy-3-oxochol-4-enic acid (conjugated).

formula $C_{28}H_{50}O_6$ required for the ethyl ester of a 'tetrahydroxynorsterocholanic acid', $C_{27}H_{48}O_6$. The possible significance of these analytical figures is discussed below. An attempt was made to effect further purification of our 'ethyl tetrahydroxynorsterocholanic acid' by partition chromatography on Celite (kieselguhr) columns, as described by Carpenter & Hess (1956); this was unsuccessful.

Oxidation by chromic acid of a less pure sample of 'ethyl tetrahydroxynorsterocholanic acid', of m.p. about 180–196°, gave a neutral mixture melting over a wide range. This material was partially separated on Celite columns, giving a pure sample of ethyl dehydrocholate (ethyl 3:7:12-trioxocholanic acid) and a substance of m.p. 157–166°, together with a mixture. A study of the result of this separation suggested that the ester, m.p. 180–196°, might consist of a binary mixture of about 57% of ethyl cholate and 43% of ethyl tetrahydroxynorsterocholanic acid: however, the presence of other constituents could not be excluded. Further purification of 'ethyl tetrahydroxynorsterocholanic acid' was not feasible with the amounts of material available.

Infrared spectra of the chromic oxidation product, m.p. 157–166°, mentioned above and of pure ethyl dehydrocholate were compared. The spectra showed a general similarity, but also well-marked differences. A comparison between the infrared spectra of methyl cholate and of a methylated sample of Kazuno's acid has already been reported (Haslewood & Wootton, 1956).

EXPERIMENTAL

General. Melting points were determined on a Kofler-block type of apparatus, and are corrected. Infrared spectroscopy was carried out as described by Wootton & Wiggins (1953). Al_2O_3 (S) was 'Type H' (Peter Spence and Sons Ltd., Widnes, Lancs.); Al_2O_3 (N) was also from Peter Spence and Sons Ltd., neutralized as described by Shoppee (1949). Light petroleum had b.p. 40–60°, unless otherwise stated. Chromic acid (20%) was a solution of about 20 g. of CrO_3 in a minimal volume of water made, with mixing, to 100 ml. with acetic acid. Microanalyses (C, H) were by Drs Weiler and Strauss, Oxford. Bile salts were isolated as described by Haslewood & Wootton (1950). 'Amyl acetate' was a commercial sample of mixed isomers. It was redistilled and then had b.p. 130–140°.

Paper chromatography of bile salts. With King penguin bile salts, Haslewood & Sjövall (1954) found spots corresponding to taurocholate and taurodeoxycholate in a system not distinguishing between taurodeoxycholate and taurochenodeoxycholate. In the present work, when the method of Sjövall (1955) for separating these substances [descending paper chromatography: mobile phase, 80% (v/v) amyl acetate–heptane; stationary phase, 70% (v/v) acetic acid–water] was used, spots corresponding to taurocholate and taurochenodeoxycholate (not taurodeoxycholate) were detected.

Preparation of crude 'bile acid esters'. King penguin crude bile salts (1 g.) were hydrolysed at 115° for 4 hr. in a

sealed metal bomb with 2.5N-NaOH (10 ml.). The acids obtained by acidification of the diluted contents of the bomb after the addition of excess of sodium chloride were washed by manipulating the usually sticky mass under water. Esterification was effected by dissolving the crude acids, freed from excess of water by decantation, in cold ethanol containing 2% (v/v) H_2SO_4 and leaving overnight. The product was isolated by ether extraction of the mixture after dilution with water. The ether was washed with aqueous $NaHCO_3$ and water, dried (Na_2SO_4) and evaporated. Yield of 'crude esters', approx. 560 mg. Acidification of the bicarbonate washings gave no unesterified acid.

Chromatography on alumina

Separation of ethyl esters. 'Crude esters', prepared as above, were dissolved in benzene (about 20 ml.) and the solution was poured onto a column (diam. 14 mm.) of Al_2O_3 . Fractions eluted by solvents of varying polarities were examined chromatographically on paper and otherwise as described. Representative results for Al_2O_3 columns of the two types used are given in Tables 2 and 3. Al_2O_3 (S) columns caused some loss of the esters, probably by hydrolysis.

Paper chromatography of column fractions. Methods previously described (Haslewood, 1954) were used. Fractions suspected of containing dihydroxy esters (eluted with ethyl acetate or ether–benzene) were run in system A (Bush, 1952). Later fractions were run in system B_2 or, more successfully, in system B_3 (Bush, 1952). Spot 'X' (Tables 2 and 3) ran at about 68% of the rate of ethyl cholate in system B_3 . Combined fractions 31–35 from Al_2O_3 (N) columns showed, in addition to spot 'X', a spot running in system B_3 at a rate slightly slower than that of 'X' and also a spot running at about 25% of the rate of ethyl cholate.

Identification of chenodeoxycholic acid. The gums obtained in fractions 4 and 5 (Table 2) were combined and dissolved in 50% (v/v) acetic acid–acetic anhydride (4 ml.); acetylation was effected by adding, with cooling, 1 drop of 8.5N-perchloric acid. After 15 min. the mixture was diluted with water and extracted with ether. Evaporation of the washed and dried (Na_2SO_4) ether left a gum which was purified by running it through Al_2O_3 (S, 5 g.) in benzene (10 ml.). The benzene-eluted material (226 mg.) crystallized from light petroleum in long colourless needles, m.p. 98–100°. Recrystallization from light petroleum raised the m.p. to 103–104°, not depressed by admixture with authentic ethyl diacetyl chenodeoxycholate (m.p. 103–104°).

Identification of cholic acid. The partly crystalline material from (combined) fractions 6–8 (Table 2) was washed with a little 25% (v/v) ether–light petroleum, and the solid (93 mg.) obtained by filtration was recrystallized from benzene–light petroleum to give needles of m.p. 163°, not depressed by admixture with authentic ethyl cholate. This ester (approx. 10 mg.) in cold acetic acid (2 ml.) was oxidized by adding, with mixing, a few drops of 20% CrO_3 at room temperature. The resultant dehydroester (recovered by dilution with water, extraction with ether and evaporation of the washed and dried extract) was recrystallized from aqueous ethanol to give needles of m.p. 213°, not depressed by authentic ethyl dehydrocholate.

Evidence for the presence of tetrahydroxynorsterocholanic acid. In an early experiment, material eluted from an Al_2O_3 (N) column with 10% (v/v) ethanol–ether was recrystallized from ethyl acetate as fine needles, m.p. 174–175° (Found: C, 71.3; H, 10.3%). This substance ran at the

same speed as ethyl cholate on a paper chromatogram in system B₅ (Bush, 1952) and had a 'fingerprint' (1200–900 cm.⁻¹) infrared spectrum almost identical with that of ethyl cholate. Hydrolysis followed by re-esterification with ethanol of this substance gave a product which, after crystallization from benzene–light petroleum, had m.p. 195–198°.

In another experiment, combined fractions [500 mg., 20% (v/v) acetone–ether eluate] from Al₂O₃ (N) columns were left for 2 days with cold ether. The crystalline residue left after decantation of ether was washed with cold ether; it then had m.p. 172–176°. This material was recrystallized from aqueous ethanol, light petroleum–acetone (twice), aqueous ethanol and benzene (in that order), finally giving white needles (14 mg.) of m.p. 196–200° (Found: C, 71.5; H, 10.7. C₂₆H₄₄O₆ requires C, 71.6; H, 10.1. C₂₆H₅₀O₆ requires C, 70.5; H, 10.1%).

This material (5 mg.) was dissolved by gentle warming and left for 2 hr. in ethanol (0.3 ml.) with 5N-KOH (2 drops) and water (1 drop). The solution was diluted with water and treated with excess of 5N-HCl and of NaCl. The gelatinous precipitate was collected, washed with water and dissolved in ethanol. Evaporation left a residue which was warmed with ethyl acetate (about 1 ml.) and filtered after cooling. Repetition of this process finally removed a persistent amorphous impurity and allowed the preparation of a clear solution in about 0.5 ml. of ethyl acetate. On standing, at

about 20° and then 5°, this deposited the bulk of the product as white needles; solvent was decanted and the crystals were washed with cold ethyl acetate. They then had m.p. 198–200°, depressed to 185–194° by a sample of cholic acid (m.p. 196–198°), and showed the same behaviour after melting as Kazuno's tetrahydroxynorsterocholanic acid (see below). In another experiment a similar hydrolysis of a sample of crystalline ester gave after treatment of the product with ethyl acetate white needles of m.p. 211–214°, not depressed by Kazuno's acid (below) and showing the same behaviour after melting as this substance.

Tetrahydroxynorsterocholanic acid, given by Professor T. Kazuno of Okayama Medical School, had m.p. 215–217°; at about this m.p. formation of a small amount of fresh crystals could be seen, and these decomposed at about 298°. Kazuno's acid (1.6 mg.) was left in solution for 2 days with 2% (v/v) H₂SO₄–ethanol (0.2 ml.). The mixture was treated with excess of aqueous NaHCO₃ and the solid ester collected and crystallized from dilute ethanol, giving white needles of m.p. 200–203°, not depressed by the above ester, [m.p. 196–200°, prepared from the 20% (v/v) acetone–ether eluates of Al₂O₃ (N) columns]. Both esters ran at the same rate as ethyl cholate on paper in Bush's (1952) system B₅; ethyl 3α:7α:12α-25D-trihydroxycoprostanate ran at more than twice this rate.

Isolation of 'ketonic' fraction. A fraction (240 mg.) corresponding to 4–5 of Table 2 was boiled under reflux for

Table 2. *Alumina chromatography (first type) of ethylated 'bile acids' from King penguin bile*

The crude esters (1050 mg., black gum) were fractionated on 30 g. of Al₂O₃ (S).

Fraction no.	Eluted by (100 ml. for each fraction)	Wt. (mg.)	Appearance	Results of paper chromatography (for methods, see text)
1, 2	Benzene	197	Oil	—
3	Ether	4	Oil	—
4	Ethyl acetate	101	Gum	Spot as ethyl chenodeoxycholate
5	Ethyl acetate	228	Gum	
6–8	Ethyl acetate	157	Partly cryst.	Spot as ethyl cholate
9, 10	Ethyl acetate	50	Solid	—
11	Ethyl acetate	16	Solid	—
12	Ethanol	102	Solid	Spot as ethyl cholate and spot 'X' (see text)
13	Ethanol	2	Solid	—
Total eluted (see text)		857	—	—

Table 3. *Alumina chromatography (second type) of ethylated 'bile acids' from King penguin bile*

The crude esters (731 mg.) were fractionated on 7.3 g. of Al₂O₃ (N) (see text).

Fraction no.	Eluted by (solvent, ml. total vol.)	Wt. (mg.)	Appearance	Results of paper chromatography (for methods, see text)
1	Benzene, 100	316	Oil	Spot as ethyl chenodeoxycholate
2–8	Benzene, 350	58	Oil	
9, 10	10% (v/v) ether–benzene, 150	19	Gum	
11, 12	20% (v/v) ether–benzene, 150	12	Gum	Spots as ethyl chenodeoxycholate and ethyl cholate
13, 14	50% (v/v) ether–benzene, 150	12	Gum	
15	Ether, 100	1	Gum	
16, 17	20% (v/v) acetone–ether, 200	152	Partly cryst.	Spot as ethyl cholate and (?) unknown substance
18–26	20% (v/v) acetone–ether, 450	48	Gum	
27–30	50% (v/v) acetone–ether, 200	41	Gum	Spot as ethyl cholate and 'X' (see text)
31, 32	Acetone, 100	28	Gum	
33, 34	10% (v/v) ethanol–acetone, 150	31	Gum	Spots as ethyl cholate, 'X' and at least 2 other compounds (see text)
35	25% (v/v) ethanol–acetone, 50	8	Gum	
36	Ethanol, 50	Trace	—	
Total eluted		726	—	—

0.75 hr. with Girard's reagent T (100 mg.) in ethanol (3.0 ml.) with acetic acid (0.3 ml.). The oily 'ketones', isolated in the usual way, weighed 65 mg. This material in ethanol (5 ml.) was warmed with 5*N*-KOH (0.5 ml.) for 0.5 hr. Dilution with water and acidification with 2*N*-HCl gave semisolid acids which were collected, washed and dissolved in a minimal amount of ethyl acetate. After 9 days solvent was decanted from crystals; the latter (about 1 mg.) had m.p. 186–192°, and from aqueous ethanol gave colourless crystals of m.p. 194–199°. These were methylated with ethereal diazomethane, and the residue, after removal of solvent, was dissolved in CS₂. In the range 1200–900 cm.⁻¹ the infrared spectrum resembled closely that of methyl deoxycholate (methyl 3 α :12 α -dihydroxycholanate) and in the region 1800–1650 cm.⁻¹ only one band was present, which was attributed to the carboxylic acid ester. The nature of this substance, evidently carried over from the 'non-ketones', has not been further investigated.

Partition chromatography

An attempt was made to separate ethyl cholate and ethyl tetrahydroxynorsterocholinate and their chromic oxidation products on Celite columns as follows. Celite (kieselguhr,

Hyflo Supercel, Johns-Manville Co. Ltd.) was washed with acid and water as described by Carpenter & Hess (1956) and then dried at 60°. For each mixture solvent systems were first found in which the partition coefficient moving phase/stationary phase was about 0.2. The stationary phase was mixed with the Celite and the whole transferred with excess of moving phase: the column was packed as described by Howard & Martin (1950). Substances to be separated were dissolved in not more than 0.7 ml. of stationary phase; the solution was run on 0.1 g. of dry Celite, lightly packed on the top of the column as soon as supernatant moving phase had drained from it. Carpenter & Hess's (1956) theoretical treatment enabled a prediction to be made of the volume (V_E) needed to elute the desired compounds, and fractions of 2 ml., embodying V_E , were collected automatically into weighed tubes. Table 4 gives details of columns; Carpenter & Hess's terms are used: V_s = vol. of stationary phase; $V_{sup.}$ = wt. (g.) of Celite/density of Celite (1.8); $\pi^2 h$ = vol. of column. The results are shown in Table 5.

Preparation and chromatography of oxidation product (Celite columns 4 and 5 of Tables 4 and 5). The mixture of ethyl esters (11 mg., m.p. 178–200°) recovered from Celite column no. 3 (Table 5) in acetic acid (0.4 ml.) was left with

Table 4. Details of partition columns on Celite

For explanation of symbols see text. The parts by volume of solvents given were mixed and equilibrated in a separating funnel. The lower phase was then used as the stationary, and the upper as the moving, phase.

Celite column no.	System (parts by vol.)	Substance put on column (mg. in ml. of stationary phase)	Wt. of Celite (g.)	V_s (ml.)	$\pi^2 \times h$ (cm. ² × cm.)	Rate of flow (ml./hr.)
1	Heptane, 5; benzene, 5; ethanol, 10; water, 4	Ethyl cholate (21 in 0.3)	9	5	1.29 × 16.4	5.2
2	As col. no. 1	King penguin ethyl esters, crystallized from benzene to m.p. about 180–196° (17 in 0.3)	9	5	1.29 × 16.4	5.2
3	As col. no. 1	As above (17 in 0.3)	36	20	1.4 × 70	11.0
4	Light petroleum (b.p. 83–95°), 4; benzene, 1; ethanol, 5; water, 1	Chromic oxidation product of the above (described in text; 10 in 0.7)	9	5	1.4 × 17.9	15.0
5	As col. no. 4	As above (11 in 0.7)	36	20	1.4 × 73	11.0

Table 5. Results of partition chromatography on Celite columns described in Table 4

Fractions of 2 ml. of effluent were collected.

Celite column no.	Vol. (ml.) of effluent (V_E) giving crystals	Eluted crystals		Character of graph: mg. eluted/ml. V_E/V_E	Remarks
		Wt. (mg.)	Approx. m.p.		
1	24–34	21	163–164°	Single sharp peak	Probably the m.p. of pure ethyl cholate
2	22–34	17	178–200°	Single sharp peak	At peak, 2 ml. fraction contained about 4 mg.
3	115–143	17	178–200°	Single sharp peak	At peak, 2 ml. fractions contained about 2 mg.
4	42–50 52–60 62–66	2.7 3.8 1.6	214–227° 140–216° 148–158°	Sharp peak with later tailing	For possible interpretation, see text
5	176–208 210–250	(A) 7.3 (B) 3.0	180–226° 156–166°	Broad peak with considerable tailing	No fraction contained more than about 0.5 mg. For further treatment of A and B, see text

20% CrO_3 (0.1 ml.) for 15 min. Excess of water and then NaCl were added, and the precipitate was extracted with ether. The ether was washed with water, aqueous NH_3 and water, and dried (Na_2SO_4) and evaporated. The residue (9.8 mg.) had m.p. about $135\text{--}195^\circ$. Chromatography of this material gave results shown in Table 5; these are further discussed below.

For the Celite column no. 4 (Table 5), if the 2.7 mg. portion, m.p. $214\text{--}227^\circ$, is taken as ethyl dehydrocholate, the 1.6 mg. portion, m.p. $148\text{--}158^\circ$, as a substance which when pure would melt at (say) about 170° , and the 3.8 mg., m.p. $140\text{--}216^\circ$, as being composed of equal weights of the two compounds, then the approximate composition of the mixture recovered from the column was: ethyl dehydrocholate ($2.7 + 3.8/2 = 4.6$ mg.), 57%; substance of m.p. about 170° ($1.6 + 3.8/2 = 3.5$ mg.), 43%.

Eluted material was further treated as follows: Fractions A (Celite column no. 5, Table 5) were combined and recrystallized from aqueous ethanol, giving long colourless needles (4 mg.), m.p. $221\text{--}223^\circ$, not depressed by ethyl dehydrocholate (m.p. $221\text{--}223^\circ$); fractions B, from the same column, similarly treated, gave fine colourless needles (2 mg.), m.p. $157\text{--}166^\circ$; these (1 mg.) were dissolved in CS_2 and the infrared spectrum of the solution in the range $1200\text{--}900\text{ cm}^{-1}$ was compared with that similarly obtained from the above specimen of ethyl dehydrocholate. There were definite differences in the spectra, in spite of an overall similarity.

DISCUSSION

Biological. The evolutionary position of penguins has been fully discussed by Simpson (1946), and the consensus of opinion appears to be that there are no adequate grounds for believing that the line of descent of these birds from reptiles differs from that of the other Carinate birds. Our findings do not conflict with this view, for there is clearly a close resemblance between the bile salts of the King penguin and those of the other birds mentioned in Table 1. The only obviously different feature is the occurrence in King penguin bile of tetrahydroxynorsterocholanic acid and probably of cholic acid in amounts considerably greater than have been found in the bile of other birds. In preliminary work we have found the same to be true for the bile salts of the Gentoo penguin, *Pygoscelis papua*. This difference may have a dietary origin; however, we think it desirable to defer further discussion of the biological significance of the differences in bird bile acids until many more species have been examined and the chemical nature of tetrahydroxynorsterocholanic acid has been elucidated.

Chemical. The most striking chemical finding in the present work is the great similarity between cholic and tetrahydroxynorsterocholanic acids and their ethyl esters. Ohta's (1939b) tetrahydroxynorsterocholanic acid was isolated from the bile of a fish, and so presumably was that used by Isaka (1940). The sample sent to us by Professor Kazuno was, we believe, obtained from chicken bile by Yamasaki (1951), who put forward the view that it is

identical with Ohta's acid. Our new material is undoubtedly substantially the same as, though less pure than, Kazuno's sample. Our analyses suggest that ethyl tetrahydroxynorsterocholanic acid may have the formula $\text{C}_{26}\text{H}_{44}\text{O}_5$; however, the analytical sample certainly contained some ethyl cholate. If the formula $\text{C}_{26}\text{H}_{44}\text{O}_5$ proves to be correct, the parent acid, $\text{C}_{24}\text{H}_{40}\text{O}_5$, is an isomer of cholic acid. Such isomerism could lie at an asymmetric centre or centres, for example, at C-17 or C-20 in the steroid nucleus. The isomerism, if it exists, is not concerned only with the nuclear hydroxyl groups (which we believe on spectroscopic evidence to be at $3\alpha:7\alpha$ and 12α : Haslewood & Wootton, 1956), for if it resided in these groups alone the product obtained by oxidation of 'ethyl tetrahydroxynorsterocholanic acid' with chromic acid would have been ethyl dehydrocholate, instead of containing, as it did, a large proportion of a quite different substance. We defer further discussion on the chemical nature of tetrahydroxynorsterocholanic acid until more material is available. It is clear that King penguin bile also contains a number of hitherto unknown substances, which may be bile acids. Examples of these are: the substance giving spot 'X' (Table 2), the two compounds mentioned in fractions 33-35 of Table 3 and the non-ketonic substance finally giving an infrared spectrum like that of methyl deoxycholate, described on p. 326.

SUMMARY

1. The bile salts of the King penguin (*Aptenodytes patagonica*) have been shown to contain chenodeoxycholic, cholic, tetrahydroxynorsterocholanic and at least four other (unidentified) acids. The proportion of 'cholic-tetrahydroxynorsterocholanic' acids was greater than that reported in the bile of other birds. Apart from this, King penguin bile salts resembled those of the other birds examined by previous workers.

2. An unsuccessful attempt has been made to separate completely by crystallization and partition chromatography the constituents of the 'ethyl cholate-ethyl tetrahydroxynorsterocholanic' mixture from King penguin bile salts. The purest sample of 'ethyl tetrahydroxynorsterocholanic' prepared by us from this mixture had m.p. $196\text{--}200^\circ$, gave analytical figures in agreement with $\text{C}_{26}\text{H}_{44}\text{O}_5$, and was hydrolysed to a mixture consisting largely of 'tetrahydroxynorsterocholanic acid', apparently identical with a sample earlier given to us by Professor T. Kazuno. Ethyl cholate, $\text{C}_{26}\text{H}_{44}\text{O}_5$, similarly recovered from partition chromatography on Celite (kieselguhr) columns, had m.p. $163\text{--}164^\circ$.

3. Chromic oxidation of impure 'ethyl tetrahydroxynorsterocholanic', m.p. $180\text{--}196^\circ$, gave a mixture from which was obtained a specimen of

pure ethyl dehydrocholate, m.p. 221–223°, and an impure but different ester, m.p. 157–166°. The infra-red spectra of these two substances in the range 1200–900 cm.⁻¹ showed an overall similarity but also definite differences.

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The Effect of Sheep-Rumen Contents on Unsaturated Fatty Acids

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Diets rich in unsaturated oils modify the depot fats of most animals to resemble the dietary fats, but ruminant depot fats are relatively unaffected (cf. Shorland, 1955). In addition, ruminant fats contain higher proportions of fully saturated glycerides than would be expected on the basis of the even or widest distribution rule of Hilditch (1956). Other peculiarities of ruminant fats include the presence of substantial amounts (5–10%) of *trans* unsaturated acids (Hartman, Shorland & McDonald, 1954, 1955) and of mono- and di-unsaturated acids which differ from oleic and linoleic acids in the position of their double bonds (cf. Shorland, 1956). Hilditch (1956) has suggested that the peculiarities of the glyceride structure of ruminant fats may be due to the hydrogenation *in situ* of preformed oleoglycerides. However, while this process may possibly occur, the most important factor is probably the

hydrogenation of the dietary unsaturated fatty acids by the rumen micro-organisms, giving rise to less-unsaturated acids and stearic acid.

Perhaps the first observation of the effect of the rumen on the dietary fat was made by Reiser (1951). He reported that linolenic acid present in linseed oil was converted *in vitro* into linoleic acid by the action of sheep-rumen contents. Similar results were obtained by Hoflund, Holmberg & Sellman (1956), using fistulated sheep.

As a result of the observation of Willey, Riggs, Colby, Butler & Reiser (1952) that the depot fats of steers feed on cottonseed oil contained more stearic acid than the controls, R. Reiser (private communication) suggested that the high content of stearic acid in the depot fats of ruminants was due to the hydrogenation of dietary C₁₈ unsaturated acids in the rumen. This suggestion was further supported