Sheep intestine

A complete length (63 ft.) yielded cholesterol containing 0·037 % 7-dehydrosteroid.

DISCUSSION

The distribution of material showing the characteristic absorption spectrum of steroid-5:7-dienes—e.g. ergosterol and 7-dehydrocholesterol is consistent with the widespread occurrence of a special dehydrogenase, the normal substrate of which is cholesterol. The enzyme is demonstrably present in the intestinal mucosae of rats and guinea pigs (Glover *et al.* 1952). Although the 7-dehydrosteroid content of the intestines is low in ox, pig, horse and sheep, the occurrence of the enzyme is probable. The substrate is most likely to be endogenous cholesterol, since the main sterols which are present in the food of herbivora are the sitosterols and stigmasterol, which are in all probability very poorly absorbed. Unpublished work from this laboratory by Miss A. Duncan and Dr J. Glover shows that in the rat absorption of plant sterols is extremely inefficient. According to Windaus & Bock (1937*b*, 1938) the 7-dehydro absorption spectrum shown by sterols from cotton-seed oil and wheat-germ oil is due to ergosterol (fully identified). From this it is clear that animals may well ingest very small amounts of ergosterol in food and perhaps inadvertently with fungal spores. It remains to be seen whether animals can dehydrogenate plant sterols or hydrogenate ergosterol. This problem is being studied.

The importance of 7-dehydrocholesterol or ergosterol resides according to present knowledge in their capacity to act as photochemical precursors of vitamin D. To stress this overmuch may be to hinder progress in elucidating the wider problem of cholesterol metabolism. It is a striking fact that one of the first detectable steps is dehydrogenation at carbon atoms 7 and 8 and that this may occur in gonads, in roe and milt and in mammalian stomach and intestine.

The fate of the 7-dehydrocholesterol will be discussed in a later paper.

SUMMARY

1. Herring roe and milt contain small amounts of material showing the absorption bands of 7-dehydrosterol and a substance with $\lambda_{max.}$ 272 mμ. in *cyclo*hexane.

2. Pigskin contains 0·002 % 7-dehydrocholesterol. The sterol from pig intestine contains small quantities of materials with $\lambda_{max.}$ 253 mμ., 265 mμ. and 272 mμ. respectively and traces of 7-dehydrosterol.

3. Horse intestine yields crude cholesterol containing 0·014–0·042 % 7-dehydrosterol. The cholesterol from stomach outer layers contains *ca.* 0·013 %.

4. The cholesterol from ox intestine contains 0·011 % and that from sheep intestine 0·037 % 7-dehydrosteroid.

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Cholesta-3:5-dien-7-one in Human Atherosclerotic Aortas

BY N. L. KANTIENGAR* AND R. A. MORTON
Department of Biochemistry, The University of Liverpool

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Several groups of workers have obtained cholesta-3:5-dien-7-one from tissues: Ruzicka & Prelog (1943) from pig testes; Hardegger, Ruzicka & Tagmann (1943) from atherosclerotic aortas; Prelog, Ruzicka & Stein (1943) from pig spleen; Daniel, Lederer & Velluz (1945) from wool fat, and Karrer & Naik (1948) from ox liver. It has been obtained in this laboratory from horse liver (Cain & Morton, 1955).

Bergstrom & Wintersteiner (1941) were able to prepare the dienone from products formed by autoxidation of colloidal cholesterol (7α- and 7β-

* Present address: Medical School, University of Mysore, Mysore, India.

hydroxycholesterol and 7-oxocholesterol). Mauthner & Suida (1896), indeed, obtained the dienone by hot saponification of 7-oxocholesteryl acetate. If, then, tissue contains 7-oxocholesterol, esterified or free, the isolated dienone could be a laboratory artifact. Daniel *et al.* (1945) saponified wool fat in the cold with sodium methoxide in anhydrous ether and obtained an unsaponifiable fraction which did not exhibit the negative optical rotation characteristic of the dienone. This suggests that in wool fat at least, cholesta-3:5-dien-7-one is an artifact.

It does not, however, necessarily follow that this is true for tissues such as atherosclerotic plaques. The matter has been put to the test by avoiding saponification with hot concentrated alkali and by minimizing contact with air during extraction.

EXPERIMENTAL

Aortas were obtained through Professor H. L. Sheehan from post-mortems conducted at the Royal Infirmary, Liverpool. After flushing with cold water, the aortas were slit open and the appearance noted. Some showed raised button-like plaques, and in others the whole surface was covered with calcareous plates in the aortic wall. The atheromatous plaques were dissected and repeatedly extracted with cold ether, freshly distilled over reduced Fe. The combined extracts were dried over anhydrous Na_2SO_4 and the solvent was removed by distillation under reduced pressure in N_2. The residue was dissolved in light petroleum (b.p. 40–60°) and the absorption spectrum measured. The material was afterwards subjected to chromatography on Al_2O_3 (P. Spence and Co., grade 0) weakened by addition of water 5% (w/w) to a cream prepared with light petroleum. The fractions obtained were examined spectroscopically.

Cholesta-3:5-dien-7-one shows in *cyclo*hexane or light petroleum $\lambda_{max.}$ 268 mμ. $E^{1\%}_{1cm.}$ 693.

RESULTS

Case I: male, aged 72 years. The patient died of coronary thrombosis and the aorta showed typical atherosclerotic changes. The dissected plaques yielded 0·586 g. of ether-soluble material, which showed a pronounced inflexion near 270 mμ. in its absorption curve (1 cm. thickness, 0·117% in light petroleum). The material was chromatographed: fraction 1, 0·366 g., eluted by light petroleum, showed no selective absorption before or after saponification, and consisted of practically pure esterified cholesterol with a little yellow colour; fraction 2, 0·027 g., eluted by 2% (v/v) ether in light petroleum, showed an inflexion at 270–280 mμ.; fraction 3, 0·030 g., eluted by 10% ether in light petroleum, showed $\lambda_{max.}$ 268 mμ., $E^{1\%}_{1cm.}$ 14·8 in *cyclo*hexane, 278 mμ. in ethanol, 310 mμ. in conc. H_2SO_4 (due probably to a substance accompanying the dienone but not identical with it) and an inflexion at 350–355 mμ. (due to dienone); fraction 4, 0·115 mg. eluted by 20% ether in light petroleum, weak maxima at 235 and 265 mμ. Ether eluted a further 0·009 g. of material showing only general absorption.

Case IV: female, aged 82 years. The post-mortem examination showed coronary thrombosis, atherosclerotic aorta, enlarged and congested liver. The posterior wall of the aorta showed heavy calcareous deposits. The ether extract (5·81 g.) showed (in ether) $\lambda_{max.}$ 420, 445, 470 mμ. $E^{1\%}_{1cm.}$ 445 mμ. *ca.* 0·15 and (in *cyclo*hexane) an inflexion near 270 mμ., $E^{1\%}_{1cm.}$ 3·3. The extract, dissolved in the minimum amount of light petroleum and left overnight at 0° gave a yellow supernatant (*a*) and almost colourless crystals (*b*). The colour in (*a*) was due to β-carotene; (*a*) was saponified quickly with avoidance of unnecessary aeration and the unsaponifiable fraction (1·764 g.) showed $\lambda_{max.}$ 270 mμ. $E^{1\%}_{1cm.}$ 2·05. The crystals (*b*) weighed 0·487 g. and showed $\lambda_{max.}$ 268 mμ. $E^{1\%}_{1cm.}$ 6·07.

The unsaponifiable matter from (*a*) was chromatographed. Light petroleum eluted 0·0185 g. of yellow material $\lambda_{max.}$ 425, 445, 475 mμ. 2% ether–light petroleum eluted 0·0085 g., $\lambda_{max.}$ 268 mμ., $E^{1\%}_{1cm.}$ 69. 10% Ether in light petroleum eluted 0·308 g., mainly cholesterol with no selective absorption and 20% ether in light petroleum eluted 0·400 g. of cholesterol with weak general absorption. Ether eluted 0·0205 g. of material with $\lambda_{max.}$ 234 mμ. and an inflexion at 270–290 mμ. and weak peaks at 420, 440 and 470 mμ. due to carotenoid. This fraction, dissolved in conc. H_2SO_4, showed $\lambda_{max.}$ 315 mμ. and an inflexion at 298 mμ., the 315 mμ. peak being due to a congener probably distinct from cholesterol. The 234 mμ. peak may be due to an $\alpha\beta$-unsaturated ketone with an additional polar grouping. Cholest-4-en-3-one shows $\lambda_{max.}$ 235 mμ. (ethanol) and 298 mμ. (H_2SO_4), but is not strongly held on alumina. From these experiments it may be concluded that atherosclerotic patches contain cholesterol (largely esterified) and cholesta-3:5-dien-7-one with small amounts of unidentified congeners and of carotenoids.

Concentration of dienone from atherosclerotic patches

Pooled material (4·75 g.) from several cases, was chromatographed on alumina (50 g. weakened by 5% water, w/w). Light petroleum (0·75 l.) eluted 3·65 g. of transparent material; 2% ether in light petroleum (0·5 l.) eluted 0·4 g., $\lambda_{max.}$ 268 mμ. $E^{1\%}_{1cm.}$ 5·75; 5% ether in light petroleum (0·5 l.)-eluted 0·42 g. of material with no selective absorption and ether eluted 0·112 g. of material with $\lambda_{max.}$ 230 mμ. and an inflexion near 270 mμ. Rechromatography of the second fraction gave a 2% ether in petroleum eluate (0·026 mg.) showing $\lambda_{max.}$ 268 mμ. (petroleum) 278 mμ. (ethanol), 310 and 355 mμ. (H_2SO_4). A third chromatographic

adsorption gave 0·0095 g. of material showing $\lambda_{max.}$ 268 mμ. $E^{1\%}_{1\,cm.}$ 90 (ethanol), with improved definition. In conc. H_2SO_4 this fraction showed $\lambda_{max.}$ 355 mμ. without the 315 mμ. peak recorded before the final chromatography. This final fraction therefore contained about 13% of cholesta-3:5-dien-7-one, since the pure substance shows $E^{1\%}_{1\,cm.}$ 693 in ethanol and the substance responsible for the 315 mμ. maximum had been removed.

Examination of brain from atherosclerotic subject

Brain tissue was obtained from post-mortems on patients who had died of atherosclerosis. The blood vessels were dissected out and the brain tissue was freed from blood clots and connective tissue. The ether-soluble material was obtained as already described and divided into two equal portions one of which was chromatographed (*a*) and the other saponified (*b*). The crude lipid (*a*) showed inflexions at 230–235 mμ., 265, 275 and 280 mμ. The unsaponifiable material showed only general absorption.

A large portion of brain yielded 2·75 g. of lipid, which was saponified and the fatty acids recovered from the soaps. The unsaponifiable material was again transparent but the selective absorption of the recovered acids had increased tenfold compared with the original lipid. The absorption was due to conjugated diene- and triene-acids resulting from alkali isomerization of unconjugated acids (linoleic and linolenic).

It was evident that brain tissue contained neither cholesta-3:5-dien-7-one nor any precursor from which it could be formed as a saponification artifact.

Examination of brain from mature rats

Lipid from brain of aged rats showed $\lambda_{max.}$ 257 mμ., but the unsaponifiable fraction showed only general absorption. The recovered acids (normal saponification procedure) showed inflexions at 260, 270 and 280 mμ. Cold saponification yielded recovered acids with feeble selective absorption at 275, 285 and 315 mμ., whilst prolonged hot saponification showed very clear intense maxima in the recovered acids at 235, 270, 280, 305 and 315 mμ. Isomerization for 30 min. at 180° with ethylene glycol and sodium ethoxide resulted in an acid mixture of iodine value 100, mean mol.wt. 368 and exhibiting maxima at 236, 270, 279, 300, 315, 348 and 375 mμ., consistent with the presence in the original brain lipid of linoleic, linolenic, arachidonic and traces of other poly-unsaturated acids.

At no stage did unsaponifiable fractions from rat-brain lipid show the absorption spectrum of cholesta-3:5-dien-7-one.

Examination of human gall-stones

No evidence for the presence of the dienone was obtained; the full results of this work will be reported later.

DISCUSSION

The negative results with brain tissue from atheromatous subjects are not only significant in themselves, they also show that the positive results with plaques from atherosclerotic aortas cannot be due to autoxidation of cholesterol during chemical examination. The material obtained by simple crystallization of plaque lipid (case IV; 5·81g.) indicated the presence of perhaps 5–10 mg. of preformed cholesta-3:5-dien-7-one. Cholesterol (mainly esterified) is, of course, the major constituent, but an unidentified compound (or compounds) closely accompanies the dienone in the separations.

Atherosclerotic plaques are peculiarly convenient for testing whether the dienone is an artifact or not; it is impossible to ascertain in the same way whether when it is obtained from liver, spleen or testes it is already present as such or, arises from a precursor. The necessary manipulation is too prolonged, but in our view the work of Cain & Morton (unpublished) on livers from worn-out horses points away from the idea that the dienone is entirely an artifact, even in liver.

The question now arises whether cholesta-3:5-dien-7-one is formed in the plaque or whether its presence reflects some more deep-seated metabolic anomaly associated with atherosclerosis or senility. To answer this question may need more detailed study of the dienone itself. For the present at any rate the dienone cannot be ruled out in considering possible metabolic sequences starting from cholesterol.

SUMMARY

1. Atherosclerotic plaques contain much cholesterol and small amounts of cholesta-3:5-dien-7-one and other unidentified congeners with traces of carotene.

2. The dienone and cholest-4-en-3-one have not been found in brain from atherosclerotic subjects or from aged rats even when unsaponifiable fractions have been obtained under conditions capable of isomerizing appreciably the unconjugated poly-ene acids of brain lipids.

3. Cholesterol gall-stones do not contain detectable amounts of dienone.

4. The evidence pointing away from the idea that the dienone is an artifact makes necessary further investigation of the metabolism of the compound.

We are indebted to Professor H. L. Sheehan for the selection of suitable material and for post-mortem reports.

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Formation of Cholesta-3:5-dien-7-one in Rats

By N. L. KANTIENGAR* and R. A. MORTON
Department of Biochemistry, University of Liverpool

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Addition of cholesterol to the diet of rats is well known to result in fatty infiltration of the liver (Chanutin & Ludewig, 1933; Sperry & Stoyanoff, 1934), but the process is not fully understood.

Dehydrogenation of cholesterol to provitamin D_3 can occur in the intestinal mucosa (Glover, Glover & Morton, 1952) on a significant though small scale.

Cholesta-3:5-dien-7-one is a potentially significant metabolite and we have here studied its formation in rats.

EXPERIMENTAL

Animals and diets. Ten healthy adult male hooded rats very similar in age and weight were divided into groups of five; one group was fed on crushed cubes (15 g./rat/day) to which 5% (w/w) arachis oil had been added; the other group was given a diet made up of 98% of the above (control) diet and 2% purified cholesterol. At the end of 60 days, the animals were killed. The cholesterol was given in the food in the ordinary way because previous trials had shown that rats refused an aqueous dispersion of cholesterol in the detergent Tween 80. In the 8th week, the cholesterol-fed rats became inactive and dull compared with the control animals.

Examination. The rats were killed ($CHCl_3$), and the abdomen laid open for inspection of the organs *in situ*. The organs were removed and the livers were tested for vitamin A and other lipid constituents, after portions had been removed for preparing sections. Unsaponifiable fractions of lipids were obtained and subjected to chromatography on alumina, followed by spectrophotometric study of the fractions obtained.

RESULTS

Tables 1 and 2 summarize the results. Cholesterol feeding reduced the total vitamin A in each rat liver by about one-third, or (expressed as vitamin/g. liver) by half. A trace of cholesta-3:5-dien-7-one was found in the normal livers ($<0{\cdot}06$ mg./rat), whereas 0·6 mg./rat was found in the cholesterol-fed animals. 7-Dehydrocholesterol was not detectable in the fractions from the chromatography of the normal liver unsaponifiable matter, but approximately 1·6 mg. was present in the appropriate fraction from the cholesterol-fed animals.

* Present address: Medical School, University of Mysore, Mysore, India.

Table 1. *Comparison of normally fed rats with similar animals given 2% cholesterol as a dietary addition*

	Normal rats	Cholesterol-fed rats
Abdomen	Normal	Appeared larger, and fuller
Intestines	Normal	Apparently normal
Liver, wet wt. (five livers)	45·3 g.	60 g.
Liver appearance	Normal size and colour, edges well defined	Enlarged, inelastic yellow spots, edges rounded (one lobe pale yellow, very soft)
	Normal histological picture	Sections stained with Sudan red, Sudan black and osmic acid
		Some cells showed heavy fatty infiltration. Much deposition of cholesterol
Aorta	Normal	No thickening, no fatty degeneration observed
Liver unsaponifiable fraction	0·275 g. (0·605% on liver wt.)	0·395 g. (0·66% on liver wt.)
$E^{1\%}_{1\,cm.}$ (325 mμ.)	71 gross (53·1 corr.)*	30 gross (24·4 corr.)
Vitamin A/rat	5500 i.u.	3662 i.u.
Vitamin A/g. liver	607 i.u.	305 i.u.

* Allowance made for irrelevant absorption see Cama, Collins & Morton (1952).