Provitamin D in Animal Tissues

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The demonstration (Glover, Glover & Morton, 1952) that reversible formation of 7-dehydrocholesterol (cholesta-5:7-dien-3β-ol) from cholesterol can occur in the intestinal wall of the guinea pig, provides the clearest picture so far available of the origin of provitamin D. The proof that dehydrogenation can occur does not, however, mean that provitamin D is always endogenous in origin; different species may differ in respect of their efficiency in carrying out the process or in their capacity to absorb related sterols such as ergosterol. Little is yet known concerning the specificity of the sterol dehydrogenase, and even if group specificity rather than absolute specificity were characteristic of the enzyme, the position of equilibrium in respect of the reversible reaction cannot be predicted.

Susceptibility to rickets seems to vary from species to species, but it cannot be stated with confidence that availability of vitamin D (whether ingested as such or formed photochemically *in vivo*) is decisive. Even if the point were established there would still be uncertainty; the limiting factor could be the amount of provitamin D absorbed or formed *in vivo*, or it could be the accessibility to ultraviolet light of a portion of the provitamin in the body. Rosenberg (1942) found no provitamin D in the preen glands of chickens, but quite substantial amounts were shown to occur in the skin of the feet. Provitamin D does not seem to be present in human sebum to any appreciable extent (Festenstein & Morton, 1952). Again, climatic or seasonal factors might be limiting in respect of the available light energy of wavelengths less than 300 mμ. Finally there is the difficulty of entry into the blood stream of irradiation products. Thus as Stewart (private communication) emphasized, it is not easy to see how ultraviolet light could penetrate fur or wool sufficiently to act upon the provitamin D of skin. The whole problem is seen in its most acute form in the case of the herbivorous animal, which so far as our present knowledge goes, has no intake of preformed vitamin D unless it is by licking irradiated lipids from wool or fur. Sheep are not known to lick themselves or other sheep. It is clear that some of the species which seem least likely to ingest or produce vitamin D are nevertheless not normally susceptible to rickets. (Although Dunlop (1954) records that very large doses (2×10^6 i.u.) of vitamin D_3 prevents or cures 'bent leg' in sheep in Scotland.)

Many questions thus remain to be answered. In the present paper, attention has been given to one variable, the occurrence of provitamin D.

EXPERIMENTAL

The 'provitamin D' absorption curve, first recorded for the minor constituent (7-dehydrocholesterol) of crude cholesterol (Heilbron, Kamm & Morton, 1927) and later shown to be characteristic of ergosterol, pure 7-dehydrocholesterol and many other steroid 5:7-dien-3-ols (Dorfman, 1953) and similar monoannular dienes is perhaps the best known and most fully verified example of specificity in the correlation of a structural feature with a partially resolved spectrum in solution. It is assumed here that the substance accompanying cholesterol and showing the characteristic absorption bands is 7-dehydrocholesterol.

Provitamin D. This has been determined spectroscopically using the characteristic absorption maxima at 293, 281·5 and 271·5 mμ. Measurements were made using a photoelectric spectrophotometer. For pure 7-dehydrocholesterol $E^{1\%}_{1\,cm.}$ 281·5 = 308 (Glover *et al.* 1952). Correction for irrelevant absorption has been made by a method making use of readings at three wavelengths such that $\lambda_2 - \lambda_1 = \lambda_3 - \lambda_2$ (Cama, Collins & Morton, 1951). For 278, 281 and 284 mμ. the relative intensities of absorption are 0·889, 0·997 and 0·832 (Glover *et al.* 1952). The correction formula is: $E_{281\,m\mu.}\,(corr.) = 3{\cdot}663\,[2E_{281\,m\mu.\,obs.} - E_{278\,m\mu.\,obs.} - E_{284\,m\mu.\,obs.}]$. Accepting the instrumental error for $E_{obs.}$ as $\pm 0{\cdot}002$ the corresponding error for $E_{corr.}$ will be $\pm 0{\cdot}018$. It is therefore advisable to adjust the concentrations of solutions examined so that $E_{obs.}$ exceeds 0·2. When this is not possible it is better to draw a freehand curve for the continuous irrelevant absorption and to estimate the 7-dehydrocholesterol content from the curve obtained by subtracting the irrelevant from the observed absorption.

Extraction of lipids. Tissues were dried by grinding with anhydrous Na_2SO_4 before extraction with ether. The ether (B.P.) was redistilled over reduced Fe before use.

Saponification. In many cases the tissue was digested with 40% (w/v) aqueous KOH (0·5 ml./g. tissue wet wt.) until the mixture became homogeneous; an equal volume of ethanol was then added and the mixture boiled for a further 15 min. After cooling, water was added (2 vol.) and the solution was extracted with an equal volume of ether followed by four further extractions with smaller volumes of ether. The combined ether extracts were washed, dried over Na_2SO_4, filtered and the solvent removed, the last traces under N_2 or CO_2.

Chromatography. The adsorbent was grade 0 alumina (Peter Spence and Co.) sometimes weakened by stirring in 5% (w/w) water under light petroleum. The material was usually dissolved in the minimum of light petroleum and solutions of ether in light petroleum were used for development or elution.

RESULTS

Frog ovaries

Morton & Rosen (1949) found the absorption peaks of 7-dehydrocholesterol in the unsaponifiable fraction from mature prehibernation frog ovaries at intensities corresponding with about 0·038% (calculated on wet weight of ovaries). The absorption bands were not seen in immature ovaries; in ripe ova (i.e. just before oviposition) they were recorded at much lower concentrations, mainly because the ova had acquired additional water and a protective layer.

A re-investigation of prehibernation frogs (*Rana temporaria*) (11 November 1948) showed 0·036% of dehydrosteroid (1·95% calculated on the unsaponifiable fraction). Pre-spawning frog ovaries (4 April 1949) showed 0·011% calculated on the ovaries and 1·3% calculated on the unsaponifiable fraction. The good reproducibility of the results in different years for the prehibernation frogs is consistent with a physiological 'level'.

Herring roe and milt. On addition of anhydrous Na_2SO_4 to milt (200 g.) a stringy product was formed which was difficult to grind; this was covered with ether and a little ethanol was added. After standing overnight, the solvent contained 5·6 g. lipid. The residual conglomerate of tissue and Na_2SO_4 was easy to mince and the resulting fine powder was extracted overnight with ether to yield a further 2 g. lipid.

The roe lipids were obtained by grinding with silver sand and anhydrous Na_2SO_4 followed by extraction with ether and ethanol–ether mixtures. Thorough grinding is essential. Both lipids showed rather ill-defined weak absorption spectra in the region 260–290 mμ.; the roe lipid showing in addition a weak band at 318 mμ. and an inflexion at 300 mμ.

Chromatography of roe lipid resulted in fractions showing absorption near 270 mμ. almost certainly indicative of conjugated triene acid (in glycerides), and small amounts of conjugated tetraene acids (in glycerides) were indicated by selective absorption near 305 and 318 mμ. Traces of yellow material accompanied the less strongly adsorbed fractions and hydroxylated carotenoids appeared in the more strongly held fractions.

The unsaponifiable fraction from milt lipid was chromatographed on unweakened alumina. The material eluted by 40% (v/v) ether–light petroleum contained a fivefold concentration of 7-dehydrosteroid. This fraction (0·39 g.) was rechromatographed to give in the fractions eluted with 40 and 50% ether–light petroleum 0·27 g. of material containing rather more than 1% of dehydrosteroid (Fig. 1).

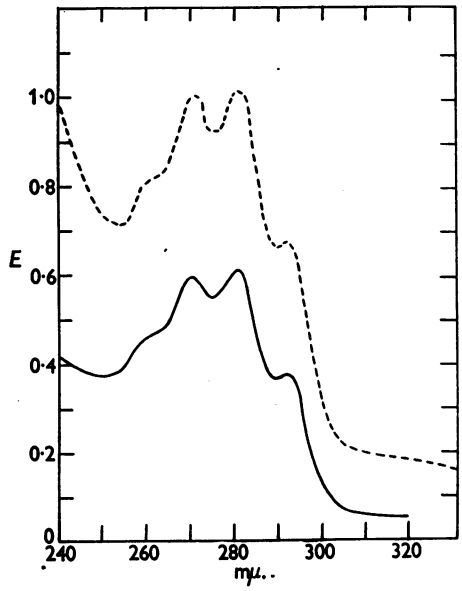

Fig. 1. 7-Dehydrosteroid fractions from chomatography of milt and roe unsaponifiable material. (Solvent, ethanol.) ——, Milt fraction (0·16%); - - - -, roe fraction (0·14%).

Similar chromatographic separations carried out on roe unsaponifiable matter gave on elution with light petroleum containing ether, a fraction in which the 7-dehydrosteroid content was nearly 2%. The overall yields of selectively absorbing sterol were: roe *ca.* 0·06%, milt *ca.* 0·04% (calculated on the unsaponifiable fraction). Windaus & Bock (1936) calculated the percentages of 7-dehydrosteroid in the cholesterol isolated from herring roe and milt as 0·12 and 0·04 respectively. Admitting the possibility of variations with the season of the year, and at the same time recognizing that losses can occur on chromatography or purification of cholesterol, the degree of agreement with Windaus & Bock seems significant. Small quantities of another substance ($\lambda_{max.}$ *ca.* 272 mμ.) were obtained in the less strongly adsorbed fractions.

Pig intestine and skin

Skin. Windaus & Bock (1937*a*) obtained 0·4–0·58 g. crude cholesterol/kg. pigskin. From 100 kg. skin, 40 g. crude sterol were obtained by them and a chromatographic fraction 0·38 g. (purity; 91% from the spectrum) was characterized through the

3:5-dinitrobenzoate as 7-dehydrocholesterol. The cholesterol from several samples of pigskin contained 2·9–5·9 % 7-dehydrocholesterol, corresponding to 0·0017–0·0035 % calculated on the weight of skin.

The estimate reached in the present work was 0·002 %.

Intestine. A complete small intestine (length 60 ft.) was washed out and external fatty tissue was cut away. Two 2·5 ft. lengths, one from each end, were subjected to alkali digestion separately and the unsaponifiable fractions examined. As no appreciable differences in spectra were shown, the remaining 55 ft. portion was treated similarly. Altogether 918 g. of tissue yielded 4·18 g. of unsaponifiable material.

Fractional crystallization of unsaponifiable matter from pig intestine. This was carried out using methanol as solvent. The least soluble fractions showed clearly the three absorption maxima characteristic of 7-dehydrocholesterol, the less soluble crystalline fractions contained small amounts of a material with a smooth absorption curve $\lambda_{max.}$ 272 mμ., whilst the mother liquors exhibited only an inflexion at 260–290 mμ. The 7-dehydrocholesterol content of the best fractions was from 0·01 to 0·02 %. The 272 mμ. chromogen fractions showed $E^{1\%}_{1\,cm.}$ *ca.* 3·0. Chromatography on alumina (weakend by 5 % water) of the residue from the mother liquors yielded very small fractions: (*a*) eluted with light petroleum; $\lambda_{max.}$ 253 mμ. (20 mg.); (*b*) eluted with 4 % ether–petroleum; weak maximum 265 mμ. (10 mg.); (*c*) eluted 6 % ether–petroleum; weak maximum 272 mμ. (6 mg.); (*d*) eluted 10 % ether–petroleum; inflexion 260–285 mμ. (14 mg.); (*e*) eluted ether; inflexions 270, 280, 290 mμ. and a trace of carotenoid (161 mg.).

Horse stomach and intestine

There are two clearly demarcated zones of the horse stomach, a glandular region and a non-glandular oesophageal region (Sisson, 1943). In our work the two portions were isolated and in each the muscle layer was separated from the outer layer.

The small intestine of the horse is about 70 ft. long and the first 3–4 ft. is regarded as the duodenum (Sisson, 1943). Portions of intestine were examined from four horses. The intestinal contents were discarded when they clearly consisted of fibrous food, but the slimy brown mucus was collected and examined separately. The portions of tissues were subjected to digestion with ethanolic KOH so as to saponify all lipid matter. The unsaponifiable matter was extracted with light petroleum instead of ether. As much cholesterol as possible was removed by crystallization from light petroleum and later ethanol or methanol. This cholesterol was bulked and recrystallized. The various samples of cholesterol were examined for ultraviolet absorption using ethanol or ether as solvent. The results are summarized in Table 1.

Table 1. *7-Dehydrosteroid* in crude cholesterol from horse intestine and stomach*

Horse	Material examined	7-Dehydrosteroid in crude cholesterol (%)
A	Intestine (first 10 ft.)	0·042
B	Intestine (first 10 ft.)	0·014
	Mucus	0·13†
C	Intestine (first 10 ft.)	0·070
	(10 ft. from caecum end)	0·027
	Stomach glandular portion:	
	Outer layer	0·012
	Muscle layer	None found
	Oesophageal portion:	
	Outer layer	0·015
D	Intestine entire length (60 ft.)	0·024

* By 7-dehydrosteroid is meant here material showing the characteristic absorption of 7-dehydrocholesterol, ergosterol and other 5:7-dien-3-ol steroids.
† High figure verified.

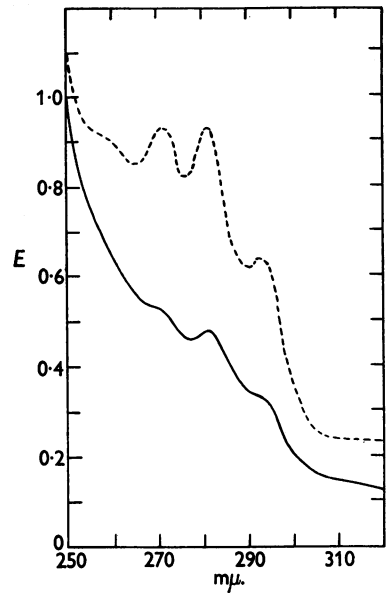

Fig. 2. 7-Dehydrosteroid in horse intestine and stomach. (Solvent, ether.) - - - - -, Duodenum (3 %); ——, ileum (2·5 %).

Ox intestine

A length of 11 ft. was examined. The cholesterol from the mucus contained 0·026 % 7-dehydrosteroid and that from the tissue 0·011 %.

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

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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-

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