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Sterol Metabolism

1. THE BIOSYNTHESIS OF [¹⁴C]ERGOSTEROL IN *CARPENTELES BREFELDIANUM* DODGE

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Ergosterol, uniformly labelled with radiocarbon (¹⁴C), was required for studies on the absorption and metabolism of sterols in the guinea pig, preliminary results on which have already been published (Glover, Leat & Morton, 1954). The preparation of [¹⁴C]ergosterol having a high specific activity has been described by Hanahan & Al-Wakil (1952). They grew the yeast, *Saccharomyces cerevisiae*, on a coenzyme A-deficient medium for 24 hr. to produce cells with a low ergosterol content, an effect observed previously by Klein (1951). The deficient cells were then returned to a coenzyme A-enriched medium for 1 hr. and later incubated with labelled acetate for 24 hr. This procedure enabled the organism readily to synthesize the normal amount of ergosterol from the added acetate without appreciable dilution with acetate derived from cell material. The final yield, however, in terms of the absolute amount of ergosterol (0.12–0.2 % dry wt.), is small, and consequently the percentage recovery of the radioactivity is low.

Ergosterol having a very high specific activity was not essential for our studies but an improved yield of the ¹⁴C recovered as [¹⁴C]ergosterol from the [*methy*l-¹⁴C]acetate precursor was sought. Previous studies in this laboratory (Glover, 1949) on the ergosterol content of moulds indicated that, under certain cultural conditions, some contained more

than 1 % of ergosterol (based on the dry weight of the mycelium), an observation in agreement with the work of Zook, Oakwood & Whitmore (1944). The mould, *Carpenteles brefeldianum* Dodge, obtained from the Centraalbureau voor Schimmelkulture, Baarn, Holland, was selected for the biosynthesis of [¹⁴C]ergosterol because (a) this organism produces at least 1.5 % of its dry weight as ergosterol with acetate in the medium, whereas *Saccharomyces cerevisiae* synthesizes less than 0.35 % under conditions for the biosynthesis of [¹⁴C]ergosterol from labelled acetate (Kodicek, 1955; Hanahan & Al-Wakil, 1952); and (b) the ergosterol is easily extracted with fat solvents from the dried thallus, whereas that in yeasts is tightly bound in the cell structure and is obtained quantitatively only after digesting the cells in strong alkali (Shakir, 1948; Shaw & Jefferies, 1953).

EXPERIMENTAL

Media and materials

Raulin-Thom medium used previously by Oxford, Raistrick & Simonart (1935) for the culture of the above mould contained excessive carbon for biosynthetic work with ¹⁴C-labelled compounds and had to be modified. Their medium and the one finally adopted by us are listed in Table 1. The chemicals used were of 'Technical' grade.

Table 1. *Growth media for the mould Carpentales brefeldianum Dodge*

Constituent	Raulin-Thom medium (g./100 ml.)	Present medium (D) (g./100 ml.)
Glucose	5.0	1.0
Tartaric acid	0.27	—
Ammonium tartrate	0.27	0.05
L-Asparagine	—	0.2
Sodium acetate	—	0.05
K ₂ CO ₃	0.04	—
MgCO ₃	0.027	—
(NH ₄) ₂ HPO ₄	0.04	—
KH ₂ PO ₄	—	0.04
MgSO ₄ ·7H ₂ O	—	0.03
(NH ₄) ₂ SO ₄	0.017	0.15
ZnSO ₄ ·7H ₂ O	0.005	0.005
FeSO ₄ ·7H ₂ O	0.005	0.005

The pure anhydrous crystalline ergosterol [$E_{1\text{cm}}^{1\%}$ (281.8 m μ) = 299] used for the standardization of the analytical methods was kindly supplied by Glaxo Laboratories Ltd., Greenford, through Drs W. H. C. Shaw and J. P. Jefferies. The [methyl-¹⁴C]acetate was obtained from Radiochemical Centre, Amersham. Its specific activity was 38 $\mu\text{C}/\text{mg}$.

The ether for extraction of the mould lipids was dried over sodium wire and distilled. The dry ether was then redistilled over reduced iron to remove peroxides.

The alumina employed for chromatography was grade 0 (Spence and Co., Widnes), which was weakened by the addition of water (2%, v/w).

Methods

Preparation of cultures. The master culture was maintained by transfer on to slants prepared from Sabouraud agar [Oxo Ltd. (Medical Dept.), London], 8 ml. in a 1 in. test tube.

Concentrated stock solutions of the inorganic compounds were prepared and stored in the cold. The various media were prepared in water and adjusted to pH 5.5, and then transferred to Roux bottles (100 ml. in each). The bottles were sterilized at 15 lb./in.² for 15 min.

For labelling experiments, sodium [methyl-¹⁴C]acetate was sterilized by passing the solution (usually 50 ml.) of appropriate concentration through a Seitz ground-glass filter. Known amounts (5–10 ml.) were then added to each Roux bottle from a sterile pipette before inoculation with the organism. Under sterile conditions the bottles were fitted with inlet and outlet tubes, to allow the ¹⁴CO₂ formed to be collected in 10% (w/v) NaOH solution by drawing a slow stream of CO₂-free air through them. A spore suspension was prepared by adding 10 ml. of sterile water to a 2–4 weeks' old slant culture; 1 ml. of this solution was added to each bottle. The bottles were then incubated, without shaking, in a dark cabinet at 28° for up to 9 days, depending on the experiment.

At the end of the growth period, usually 5 days (the optimum time for ergosterol production), the thalli were collected from the medium by filtration through a sintered funnel (porosity 4), rinsed with water and freed from residual water by suction.

Extraction of the thallus. The thallus was triturated with a small amount of ethanol (5 ml./g. wet wt.) to break up the

cells and denature the proteins. The slurry was filtered through a sintered funnel at the pump and washed with a little ethanol. The cell debris was then extracted in a 250 ml. beaker five times with 50–100 ml. portions of boiling peroxide-free ether. The supernatant extracts were cooled and decanted through the filter. The bulked extracts were washed in a separating funnel twice with water to remove the ethanol and then dried over anhydrous Na₂SO₄ for 0.5 hr. The dried extract was decanted into a 250 ml. round-bottomed flask and the residual Na₂SO₄ rinsed twice with ether. The extract and rinsings were evaporated to 5–10 ml. under N₂ at low pressure (water pump) and at 28°. The lipid concentrate was then quantitatively transferred to a tared 25 ml. conical flask, the residual solvent was removed in a stream of N₂ and the lipid was weighed. The extracted residue was dried in an oven at 105° and weighed.

Saponification of the cell debris and extraction of the resulting solution with ether, or further extraction of the debris with several portions of boiling ethanol, removes a little additional lipid material, none of which, however, contains ergosterol.

Purification of ergosterol. The lipid was saponified with 10% (w/v) ethanolic KOH and the unsaponifiable matter recovered by a procedure described previously (Glover, Glover & Morton, 1952). The sterol, after further purification by precipitation from ethanolic solution as the digitonide, was collected and washed by centrifuging; the sterol was regenerated by the procedure of Schonheimer & Dam (1933), chromatographed on an alumina column as described later and crystallized from methanol.

Small amounts of the sterol were also examined by the reversed-phase paper technique of Kodicek & Ashby (1954). In preliminary experiments the residual glucose in the media was determined at various stages of growth by the standard Benedict's titration procedure or by the Folin-Wu method for glucose values below 0.1 mg./ml.

Chromatographic procedures

A column (4 cm. × 16 mm.) of 2% water-deactivated alumina was prepared and the ergosterol was placed on it in the minimum volume of a 2% (v/v) solution of ethyl ether in light petroleum. Elution of the sterol from the column was achieved by gradually increasing the proportion of ether in the developing solvent according to the procedure of Cookson, Glover & Leat (unpublished observations).

For the reversed-phase technique of Kodicek & Ashby (1954), Whatman paper no. 3, coated with liquid paraffin, was employed as the stationary phase. The R_F value (0.56) obtained for ergosterol was identical with that reported by the above workers. A modification of this method was employed for separating free and esterified ergosterol; the mobile phase was 2% (v/v) water in ethanol (Green, 1955).

Spectrophotometry

The absorption spectra of the ergosterol and other lipids dissolved in spectroscopically pure ethanol were recorded on a Beckman, Model DU or a Unicam, SP. 500, quartz ultraviolet spectrophotometer. The ergosterol in the lipid extracts was assayed with a three-point correction procedure similar to that adopted by Glover, Glover & Morton (1952) for 7-dehydrocholesterol. The wavelengths, 278.3, 281.8 and 284.3 m μ , suggested by Shaw & Jefferies (1953) were suitable.

Table 2. *Production of ergosterol with time in the mould *Carpenteles brefeldianum* Dodge*

Results represent the values obtained for a single Roux bottle containing 100 ml. of medium incubated at 28°. In Expt. 1 the normal medium D was employed, but in Expt. 2 the medium (E) contained only 0.5 % of glucose.

Time after inoculation (days)	Dry wt. of thallus (mg.)	Total lipid (mg.)	Ergosterol		
			(mg.)	(% of total lipid)	(% of total dry wt.)
			Expt. 1		
2	260	17.7	1.4	8	0.56
3	510	39.5	3.9	10	0.78
4	450	28.7	4.6	16	1.02
5	390	23.8	5.3	22	1.35
6	330	22.3	5.3	24	1.58
9	260	13.9	4.7	34	1.81
			Expt. 2		
1	9.9	0.8	0	0	0
2	250	7.9	0.8	10	0.32
3	250	8.3	1.5	20	0.59
4	190	7.6	2.2	29	1.16
5	140	5.9	2.2	37	1.57
7	130	5.0	2.2	44	1.69
8	130	5.1	2.2	43	1.75
9	130	5.2	1.9	35	1.43

Assay of radioactivity

The CO_2 resulting from the metabolism of some of the labelled acetate was absorbed in 10 % (w/v) carbonate-free NaOH solution and precipitated as $\text{Ba}^{14}\text{CO}_3$ by a standard procedure (Glover, 1956). An equimolar amount of NH_4Cl equivalent to the alkali present was first added, however, to lower the pH. This prevented excessive absorption of CO_2 from the air during the inevitable short periods of exposure of the alkaline solution during the precipitation and collection of the $\text{Ba}^{14}\text{CO}_3$. A portion as a slurry in ethanol was transferred to a 0.75 in. diameter stainless-steel cupped planchet to make a layer not thicker than 5 mg./cm.² It was then dried to form an even film under an infrared lamp and assayed at 4 % geometry with a thin end-window Geiger-Müller counter.

The ergosterol and other lipid fractions were assayed by applying 0.2–0.4 ml. of a suitable solution of the substance in chloroform to the planchet, previously prepared with a disk of lens paper (Green's no. 105) covering the base. The paper ensures an even and reproducible spread of the lipid over the dish. The various results were corrected for background and self-absorption in the usual manner. The total number of disintegrations recorded for each sample was always such as to ensure a probable error of less than 1 %.

The radioautographic procedure for detecting the labelled material on paper chromatograms has been described previously (Glover, Kamen & Genderen, 1952). Ilford Red Seal (Ilford Ltd., London) or Kodirex (Kodak Ltd., London) no-screen film was used.

RESULTS AND DISCUSSION

The original Raulin–Thom medium used by Oxford *et al.* (1935) for *Carpenteles brefeldianum* Dodge was not suitable for labelling experiments with ^{14}C , mainly because of its varied and excessive carbon components. A number of changes were made in turn to this medium: (a) the glucose concentration

was reduced from 5 to 1 % (w/v); (b) the potassium carbonate was eliminated and the potassium replaced as phosphate; (c) the tartaric acid in the medium was replaced by 0.2 % L-asparagine, which initiated growth more quickly; (d) the ammonium tartrate level was lowered to 0.05 %. The amounts of inorganic salts added were also reduced (see Table 1).

These changes permitted the addition of 0.05 % sodium acetate, the fraction carrying the labelled carbon, without affecting the growth of the organism. The production of ergosterol with time was studied with the modified medium (D) and the same medium but with only 0.5 % of glucose (medium E). Six Roux bottles each containing 100 ml. of medium D and eight similar bottles of medium E were inoculated and placed in a dark incubator at 28°. After 1, 2, 3, etc., days they were removed for examination. Each thallus was separated off and the lipids were extracted as described above; the lipids were saponified and ergosterol was determined in the unsaponifiable matter. The results are given in Table 2. This shows that the dry weight of the thallus and the total lipid content are maximal on the third day after inoculation and decline steadily thereafter, whereas the ergosterol content does not reach its maximum level until the fifth day and remains about the same until the eighth or ninth day. Examination of the medium D for residual glucose showed that on the third day after inoculation only 86 mg. of glucose remained and on the fourth day less than 10 mg. In medium E less than 5 mg. was present on the third day. Thus the peak in dry weight corresponds, as might be expected, with the exhaustion of the glucose from the medium. The rapid decline in dry weight and total lipid from the third to the fifth or sixth day is

indicative of the utilization of some cell material for the maintenance of respiration and possibly also for the synthesis of the additional ergosterol.

In four experiments with the 1% (w/v) glucose medium (D) containing 0.05% sodium acetate, the yield of ergosterol from the cultures was 4–5 mg./bottle (1.4–1.6% of the dry wt. of the mycelium).

Oxford *et al.* (1935) reported that a considerable amount of the ergosterol synthesized on the Raulin-Thom medium was in the form of the palmitate. Our experience confirms this. If the same thing occurred with our medium then direct chromatography of the extracted lipid and crystallization of the fractionated ester would have enabled us to purify the ergosterol readily. The gross lipid from our cultures was examined for the esterified form but none was detected. Esters were absent, presumably because our modified medium is much poorer as a carbon source. Although the ergosterol was free, we also saponified the lipid in order to minimize the precipitation of contaminants along with the fine ergosterol digitonide. No loss of the sterol is incurred in the saponification process.

The various preparations of ergosterol, recovered from the digitonide and recrystallized from methanol after chromatography on an alumina column, had $E_{1\text{ cm.}}^{1\%}$ (281.8 m μ) values in the range 250–276. Further recrystallization, particularly of the less-pure specimens, yielded material having $E_{1\text{ cm.}}^{1\%}$ (281.8 m μ) values in the range 270–276. Further attempts at purification caused severe oxidative losses. Pure anhydrous ergosterol obtained from Glaxo Laboratories Ltd., assayed by us under conditions identical with those for our material, had an $E_{1\text{ cm.}}^{1\%}$ (281.8 m μ) value of 299, but a figure of 306 has been reported for similar material (Shaw & Jefferies, 1953). The sterol recrystallized from methanol, however, usually contains from 1 to 1.5 molecules of water, making an $E_{1\text{ cm.}}^{1\%}$ value of 286, the maximum for such a preparation (Shakir,

1948; Shaw & Jefferies, 1953). The ergosterol from the mould therefore would appear to contain an additional 3% of transparent impurity. The lower extinction value observed, however, could be due in part at least to additional moisture and some oxidation of the material during assay. The best ergosterol preparations are generally only obtained when the recrystallization is carried out on a larger scale than in this work and when the material is handled under nitrogen.

Our preparations are comparable in purity to those obtained by Hanahan & Al-Wakil (1952), but to check that no saturated sterol was present as the impurity some ^{14}C -labelled material was chromatographed by the reversed-phase paper method on 1.5 in. strips. The chromatogram was divided into 1 cm. pieces along its length and examined for radioactivity and for ergosterol by the Liebermann-Burchard test. Only one zone, having the R_f of ergosterol, possessed radioactivity and this also gave the correct 'fast-reacting' colour test (Moore & Baumann, 1952). Radioautographs of a duplicate chromatogram confirmed the identity of the distribution of radioactivity with the intensity of the colour test.

A number of experiments were carried out with [*methyl*- ^{14}C]acetate added to the medium. The observed distribution of the radioactivity in one experiment is given in Table 3 (A). Just over one-half of the radiocarbon is lost as carbon dioxide and of the remainder approximately 20% appears in the lipid fraction. The results of the experiments in Table 4 actually show a range from 10 to 30 for the percentages of total radioactivity incorporated into the lipid fraction. These variations are partly explained by the details of the distribution of the radiocarbon in the various lipid fractions, namely, the fatty acids, 3 β -OH sterols (mainly ergosterol) and the residual unsaponifiable matter not precipitated by digitonin. The variations appear to arise mainly from the wide fluctuation in absolute amount of the fatty acids and the unsaponifiable matter not precipitated by digitonin. The sterol fraction, on the other hand, remains fairly constant. This finding is not unexpected since the cultures were always harvested at a convenient period, 5 ($\pm$ 1) days after inoculation. Table 2 shows clearly that in this period the ergosterol content of the organism remains reasonably constant, whereas the amount of other lipids changes markedly.

The final column in Table 4 indicates the degree of purity of the sterol, as recovered from the digitonide and purified by chromatography and one crystallization from methanol.

The nature of the digitonin-soluble fraction of the unsaponifiable matter has not yet been elucidated. It is strongly adsorbed on alumina but runs as a homogeneous zone in the paper chromatogram,

Table 3. *Distribution of radioactivity in various fractions of cultures of Carpentales brefeldianum Dodge grown on medium D to which [methyl- ^{14}C]acetate was added from the outset (A, controls) and after growth for 3–5 days (B)*

Four Roux bottles were pooled for each group; the cultures were grown at 28° with CO₂-free air drawn over them.

	Percentage of total ^{14}C added	
	A	B
Dried thallus (lipid-free)	7.5	16.1
Aqueous fraction from thallus	0.5	0.2
Total lipids	19.5	3.4
Residual medium	20.1	15.4
Evolved CO ₂	52.4	64.9

Table 4. *Distribution of radioactivity in the lipid fractions from Carpentales brefeldianum Dodge grown on a medium containing [methyl- ^{14}C]acetate*

Five Roux bottles were used in each experiment. The final column indicates the purity of the material after one crystallization.

Distribution of radioactivity in lipid fractions							
[Methyl- ¹⁴ C]- acetate added (μc)	Radioactivity incorporated in lipids (%)	Fatty acids (μc)	Unsaponifiable matter				Extinction of Δ ^{6:7} -sterol [E _{1cm.} ^{1%} (281.8 mμ)]
			Not precipitated with digitonin (μc)	Ergosterol			
				Activity (μc)	Wt. (mg.)	Specific activity (μc/mg.)	
32	17.5	3.5	0.4	2.0	15.5	0.13	261
60	20.5	9.4	2.1	1.9	17.3	0.11	270
211	15.5	15.0	13.5	5.2	17.3	0.3	276
85	30.0	14.8	9.2	1.9	—	—	256
118	11.4	1.8	8.7	2.8	21.2	0.13	240
70	36.8	19.6	2.6	3.3	8.5*	0.39	261

* Low yield of [^{14}C]ergosterol obtained in this experimental run was probably due to a poor inoculum.

with an R_p value (0.8) much higher than that of ergosterol (0.56). It does not show selective absorption in the visible or ultraviolet regions of the spectrum.

The results in Table 4 indicate that the yield of radiocarbon as uniformly labelled [^{14}C]ergosterol from a given quantity of [methyl- ^{14}C]acetate varies from 2 to 6%. If it is assumed that the acetate carbons added at the outset become uniformly diluted in the metabolic pool of C_2 units formed from the other carbon components in the medium, then the specific activity of the ergosterol, in $\mu\text{C}/\text{mg.}$ of carbon, should be only a little more than the total radioactivity, in μC added, divided by the total carbon content of the medium (500 mg.), since the contribution of the methyl group of acetate to the sterol structure is greater than that of the carboxyl group. The fact that it is very much higher indicates that there is a preferential incorporation of the [methyl- ^{14}C]acetate into the molecule.

This is supported by the fact that whereas the lipid fraction forms only 6–7% of the total dry weight, the amount of radiocarbon entering this fraction varies from 11.4 to 36.8%. The yield appears to be considerably higher than that obtained by Hanahan & Al-Wakil (1952) with the yeast *Saccharomyces cerevisiae*. From their published figures it can be calculated that the total incorporation of radiocarbon from labelled acetate into the sterol fraction is only about 0.003%, whereas the specially treated coenzyme A-deficient culture gives a yield of approximately 0.3%. To set against this, however, is the fact that the specific activity of our preparation is much lower.

It was thought that it might be possible to improve on this by attempting to restrict the synthesis of ergosterol during the growth of the

cellular structure and then to allow the remainder to be synthesized in the presence of labelled acetate. The method, employed by Hanahan & Al-Wakil (1952) for yeast, is unsuitable for moulds which synthesize their own cofactors. Another possibility is the use of an inhibitor; Klein, Eaton & Murphy (1954) showed that the production of ergosterol was reduced in *S. cerevisiae* grown in an atmosphere of nitrogen. The normal sterol content could be restored by transferring cells to a 1% glucose medium without a nitrogen source and aerating. A similar type of experiment has been attempted with *C. brefeldianum*. Several cultures were allowed to commence growing normally for 2 days and then oxygen-free nitrogen was passed through the bottles for 3 days. The mycelial mats of the test group and controls were not substantially different in their sterol content, so these preliminary trials indicated that this method was not applicable to *C. brefeldianum*.

Finally, it was thought that if [^{14}C]acetate were added after most of the glucose had been used up and just before the period of rapid development of the ergosterol, e.g. on the third day, then it might enter preferentially the latter fraction. In an experiment with two groups of 4 Roux bottles (Table 3) the labelled acetate was added 3.5 days after inoculation, but the yield of [^{14}C]ergosterol was inferior to that in the control group and a much greater portion (65%) of the labelled carbon was evolved as $^{14}\text{CO}_2$. Future work with selective inhibitors such as pantoyltaurine may improve the above yield, but the material produced by the above method proved suitable for a number of studies on the absorption and metabolism of ergosterol and it may be prepared with a sufficiently high specific activity for the manufacture of [^{14}C]ergocalciferol (Kodicek, 1955).

SUMMARY

A method for the biosynthesis of [^{14}C]ergosterol from sodium [methyl- ^{14}C]acetate in the mould *Carpenteles brefeldianum* Dodge is described. The specific activity of the [^{14}C]ergosterol obtained was 0.1–0.3 $\mu\text{C}/\text{mg.}$, and the total yield of radiocarbon was 2–6 %. The specific activity of the [methyl- ^{14}C]acetate used before dilution was 38 $\mu\text{C}/\text{mg.}$

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Sterol Metabolism

2. THE ABSORPTION AND METABOLISM OF [^{14}C]ERGOSTEROL IN THE GUINEA PIG

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The absorption of ergosterol has been studied in many species because its ultraviolet-absorption spectrum forms an excellent label. The early work of Schonheimer, Behring & Gottberg (1932) on the dog and Menschick & Page (1932) on the laying hen suggested that although the sterol was poorly absorbed, small amounts did in fact penetrate the intestine. The work of Schonheimer and co-workers (Schonheimer, 1931), on rats, rabbits and mice, of Cook (1938) on the guinea pig and of many other workers since then have, however, clearly demonstrated that phytosterols in general, including ergosterol, are poorly absorbed. The actual amounts which cross the intestine have only recently been assessed by using isotopically labelled sterols. Hanahan & Al-Wakil (1953) showed that 4 % of a single dose of [^{14}C]ergosterol is absorbed by the rat, and Gould (1954) has shown that tritium-labelled β -sitosterol is absorbed to the extent of 0.4 %. Similarly, 4 % of a dose of ^{14}C -labelled sterols from tomato leaves has been found to be absorbed by the guinea pig (Duncan, 1954).

The present studies on the guinea pig, which were begun some years ago, before the use of ^{14}C -labelled materials, were undertaken because: (a) the guinea pig, which possesses in the gut wall a high concen-

tration of the analogous animal sterol, 7-dehydrocholesterol ($\Delta^5:7$ -cholestadien-3 β -ol), might absorb ergosterol better than other species; if the ergosterol were hydrogenated to 7:8-dihydroergosterol (brassicasterol) or to 5:6-dihydroergosterol by the intestinal enzymes, in a manner similar to the hydrogenation of 7-dehydrocholesterol (Glover, Glover & Morton, 1952), it would escape spectroscopic detection; (b) the different steric configuration of the side chain of ergosterol could perhaps enable it to act as an inhibitor of the sterol dehydrogenase enzyme system leading to a disturbance of the normal equilibrium obtaining there; (c) ergosterol can be labelled with ^{14}C easily so that its detailed metabolism may be studied.

A preliminary account of the work has already been published (Glover, Leat & Morton, 1954).

EXPERIMENTAL

Materials. Crystalline ergosterol was obtained from Peboe Ltd., Liverpool. Uniformly labelled [^{14}C]ergosterol was prepared biosynthetically from [methyl- ^{14}C]acetate in the mould *Carpenteles brefeldianum* Dodge (Glover & Leat, 1957).

The sterol-free diet for the guinea pigs was prepared by mixing solvent-extracted food cubes (diet no. 4, British Extracting Co. Ltd., Bromborough) with 5 % of arachis oil.