75. THE STEROL REQUIREMENTS OF THE LARVA OF THE BEETLE, *DERMESTES VULPINUS* FABR.

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HOBSON [1935] showed that blowfly larvae required a fat-soluble growth factor which was contained in the unsaponifiable ether-soluble fraction of their food and proved to be identical with or replaceable by cholesterol. Subsequently the indispensability of cholesterol for the growth of many insects was demonstrated (Van t'Hoog [1936] for *Drosophila*; Chiu & McCay [1939] for the bean weevil *Acanthoscelides obtectus*; Fröbrich [1939] and Offhaus [1939] for the flour beetle *Tribolium confusum*; and for the edible snail, *Helix pomatia*, Howes [1937]). In a study of the nutritional requirements of the beetle *Dermestes vulpinus*, carried out in this department, Gay [1938] showed that growth of the larva was satisfactory on a diet consisting of casein, cystine, yeast, salt mixture and cholesterol, or of yeast, salts and cholesterol, while omission of cholesterol from either diet prevented growth. It has been the purpose of the present investigation to obtain more detailed information about the sterol requirements of this beetle.

EXPERIMENTAL

The technique of breeding was very similar to that employed by Gay [1938]. The method of obtaining eggs and newly hatched larvae has been described by Dick [1937]. The insects were bred at 27° and 70 % relative humidity except in the last series of experiments (Tables 5-7), when the temperature was 25°. It was at first thought that the larvae would attack each other and to prevent such loss of animals by cannibalism they were bred singly in glass tubes, the history of growth of each larva being followed, as a rule by daily inspection, until pupation or death. This precaution, however, later proved unnecessary and in the last series five larvae were reared together in a tube.

The following diets were used:

Diet I (called subsequently 'casein diet'): 3.0 g. ashless vitamin-free casein (casein E, Glaxo Laboratories), 0.3 or 0.15 g. dried debittered brewer's yeast (Glaxo Laboratories), 0.1 g. sterol, 0.02 g. cystine (B.D.H.), 0.03 g. MacCollum's salt mixture, distilled water to 50 % by weight.

Diet II (called subsequently 'yeast diet'): 3.0 g. dried debittered brewer's yeast (Glaxo Laboratories), 0.1 g. sterol, 0.03 g. MacCollum's salt mixture, distilled water to 50 % by weight.

Diet III (described on p. 713).

The following sterols were tested, their source being indicated in the list:

From Glaxo Laboratories: cholesterol, sitosterol, ergosterol, calciferol, 7-keto-cholesteryl acetate, 7-hydroxycholesterol, 7-hydroxycholesteryl dibenzoate, 7-dehydrocholesteryl monobenzoate, 7-dehydrocholesterol.

From Prof. I. M. Heilbron and Dr E. R. H. Jones: cholesterol purified via the dibromide, cholestanol (dihydrocholesterol), zymosterol, Δ^5 -cholestene, cholesteryl acetate.

At a temperature of 27 or 25°, a humidity of 70 % and a water content of 50 % the diet would be expected to become infested with moulds and other micro-organisms within a few days. This might seriously alter the composition of the food, apart from introducing new food factors supplied by the micro-organism. Serious efforts were made to limit the degree of infestation and four different methods were tried for this purpose.

1. An attempt was made to grow the larvae under sterile conditions. The eggs were sterilized by immersion in a 3 % solution of HgCl_2 for 3 min. The food was sterilized by heating the dry constituents, minus the yeast, at 145° for 1½ hr., and to them was added a suspension of yeast in water which had been brought to the boil. Two days later this mixture was again heated at 100° for 10 min. While the cultures seemed to remain sterile for about 12–16 days, almost invariably moulds then began to grow, most probably due to infestation from outside. Although it seemed likely that lasting sterility could be achieved by further improvement of the technique the drawbacks of the method were so manifold that it was finally abandoned.

2. During the past few years increasing use has been made of the antiseptic properties of the esters of *p*-hydroxybenzoic acid for keeping organic media free from bacteria and moulds [e.g. Cremer, 1935]. The methyl ester, known under the name 'Nipagin M', is used in this connexion in the breeding of *Drosophila*. We found in a series of experiments that admixture of 0.5 % of Nipagin in the wet casein diet prevented growth of moulds in a varying proportion of the cultures while having no influence on the growing larvae. On the other hand, moulds never appeared in a yeast diet to which 0.5 % Nipagin had been added. In spite of the uncertain effect of Nipagin as a fungicide, useful results could be obtained with this method.

3. The food was changed daily to prevent the development of moulds. This method was previously used by Gay [1938] and was adopted for the first of the present series of experiments. Although it yields very reliable results it has the disadvantage of being very laborious and requiring large amounts of the diets.

4. After many of the experiments had been completed the following diet was devised, based on the strong hygroscopic properties of fructose:

Diet III (called subsequently 'yeast fructose diet'): 1.5 g. dried debittered brewer's yeast (Glaxo Laboratories), 1.5 g. fructose, 0.05 g. sterol (or less), 0.03 g. MacCollum's salt mixture (not essential and usually omitted), 0.4 ml. water. This quantity was enough for five larvae, which were reared together in one tube.

Owing to the hygroscopic properties of fructose the diet, under the conditions at which it was used (25° and 70 % relative humidity), acquired a water content of 20 to 25 %, and the high sugar content prevented growth of moulds very effectively. One of the minor disadvantages of the diet was that the water content was not exactly the same in different samples, and since in *Dermestes* the rate of growth is very much dependent on the water content of the diet [Gay, 1938] any minor differences which were recorded in the duration of larval life could not be regarded as significant. An attempt to breed *Dermestes* on a casein diet to which 50 % fructose was added proved less successful because the diet got so wet that many young larvae were drowned in it.

In all experiments except the later ones on the fructose diet, the development of each individual larva was observed up to the time of pupation, and the subsequent emerging of the adult and the number and time of each moult were recorded. The data presented as a measure of the efficiency of a diet are limited in this paper for the most part to the times until the larvae pupated or, in case of an unsuccessful diet, died. The duration of larval life is the only figure of

significance, since growth takes place only during that period. Once a larva has pupated, the duration of the pupal stage is constant (about 7 days at 27°). The adults were always fertile. The controls were reared on fishmeal which was moistened to contain 50% water.

The first question to be decided was whether the positive effect of cholesterol on growth was not due to the presence of impurities such as 7-dehydrocholesterol, the natural provitamin D₃ which is considered to be present in commercial cholesterol. Series I compares the effect of commercial cholesterol (B.D.H.) with a sample of cholesterol specially purified (via the dibromide), which was free of 7-dehydrocholesterol. Although for reasons not connected with the course of the experiment this series had to be abandoned after 11 days, it was by then clear that the larvae grown on the two samples of cholesterol hardly differed with regard to mortality and speed of development and were slightly more advanced in development than the controls (Table 1). In the absence of cholesterol, development was slow, the mortality was high and the few surviving larvae were in an obviously dying condition.

Table 1

No.	Diet	Condition after 11 days	
1	Fishmeal (control)	9 in 4th instar	1 dead
2	Yeast diet, commercial cholesterol	9 in 5th instar	1 dead
3	Yeast diet, purified cholesterol	6 in 5th instar 4 in 4th instar	0 dead
4	Yeast diet, no sterol	2 in 3rd instar 1 in 4th instar	7 dead

Temp. 27°. Food changed daily.

Series II (Table 2) was devised to compare the effect of purified cholesterol with those of sitosterol, ergosterol and calciferol. The first three sterols were incorporated in a casein diet containing 5% yeast and the food was changed daily. This method was not used with calciferol, owing to the expense involved; the Nipagin method was used instead.

It can be seen in Table 2, nos. 7-9, that the diets containing ergosterol or calciferol were entirely inadequate. In fact the larvae died as rapidly as if no sterol had been present [cf. Gay, 1938]. Sitosterol proved of slight nutritional

Table 2

No.	Diet	Method	No. of larvae	No. pupating	Mean time to pupation days
5	Pure, cholesterol, casein diet, 5% yeast	Food changed daily	10	5	39.8
6	Sitosterol, casein, diet, 5% yeast	Food changed daily	10	Last larva died after 30 days (one pupated 47th day)	18.2
7	Ergosterol, casein diet, 5% yeast	Food changed daily	10	Nil. Last died after 13 days	10.4
8	Calciferol, casein diet, 5% yeast	0.5% Nipagin; 3 tubes grew mould 11th day	15	Nil. Last died after 15 days	12.3
9	Calciferol, yeast diet	0.5% Nipagin; no mould	15	Nil. Last died after 14 days	10.1

Temperature 27°.

value. Only one larva reached the pupal stage after long delay and the remaining larvae lived considerably longer than on ergosterol and calciferol. The course of the experiment on pure cholesterol was not entirely satisfactory. The mortality was somewhat higher than it should have been and the time to pupation of 39.8 days compares unfavourably with Gay's figure of 29.2 days, which was obtained, however, in experiments with commercial cholesterol.

Series III (Table 3) was devised to compare the efficiencies of commercial and pure cholesterol. To make the general conditions more favourable a casein diet with 10% (instead of 5%) of yeast was used. It was desirable to know whether any fat-soluble factors in yeast other than sterols would play a role in the

Table 3. *Series III. Casein diet with 10% yeast. 0.6% Nipagin added; food changed once on the 15th day*

No.	Diet	No. of larvae	No. pupating	Mean time to pupation, days
10	Commercial cholesterol	15	11	26.0
11	Pure cholesterol	15	11	26.0
12	Commercial cholesterol ether-extracted yeast	15	11	29.2
13	Pure cholesterol ether-extracted yeast	15	14	28.6
			Last died after, days	Mean time to death, days
14	Ergosterol	15	31	15.8
15	Sitosterol	15	34	20.0

Temperature 27°.

nutrition of *Dermestes*. The effect of ether-extracted yeast (extracted for 7 days at room temperature, ether changed once or twice daily) was therefore compared with that of ordinary yeast. The series was set up with the Nipagin method, and in order to obviate possible water loss from the diet it was changed once on the 15th day. In order to make the conditions strictly comparable for all the sterols used further tests with ergosterol and sitosterol were included.

Comparing nos. 10 and 11 and nos. 12 and 13 it is obvious that there is no difference in the effects of commercial and of pure cholesterol. It is therefore clear that the effective substance for the growth of *Dermestes* is cholesterol and not 7-dehydrocholesterol. It is not likely that any ether-soluble constituents of yeast are required. The slight prolongation of larval life on a diet containing ether-extracted yeast is not considered to be significant and may well be due to traces of the ether remaining in the yeast. Ergosterol and sitosterol again had no effect. Growth was very slow and all larvae died long before reaching the state of pupation, although single larvae remained alive for a considerable period. The longer survival on sitosterol and ergosterol and the quicker rate of development on cholesterol seen in Table 3, compared with those seen in Table 2, are almost certainly due to the larger amount of yeast in the diet.

In the experiments in which the food was mixed with Nipagin and was not changed daily some growth of mould usually occurred. In some tests only a small proportion of the tubes developed mould, in others a large proportion, though in none was the growth strong and it was always delayed in appearance. The data given in Table 4 show that the slight growth of mould that occurred had no significant effect upon the results of the experiments. In most instances the mean time until death or pupation of all larvae was not significantly different

Table 4. *Casein diets (except no. 9)*

No.	Medium	No. of tubes	No. with mould	Mean time to appearance of mould days	Mean time to death or pupation of all larvae days	Mean time to death or pupation of those exposed to mould days	
9	Calciferol, yeast diet, 0.5% Nipagin	15	0	—	10.1	—	Time to death
8	Calciferol, 5% yeast, 0.5% Nipagin	15	3	10.0	12.3	12.0	
15	Sitosterol, 10% yeast, 0.6% Nipagin	15	5	12.0	20.0	17.4	
14	Ergosterol, 10% yeast, 0.6% Nipagin	15	2	19.0	15.8	20.0	
11	Specially purified cholesterol, 10% yeast, 0.6% Nipagin	15	13	11.0	26.0	25.8	Time to pupation
10	Commercial cholesterol, 10% yeast, 0.6% Nipagin	15	10	9.0	26.0	25.8	
13	Specially purified cholesterol, 10% ether-extracted yeast, 0.6% Nipagin	15	5	16.6	28.6	29.2	
12	Commercial cholesterol, 10% ether-extracted yeast, 0.6% Nipagin	15	9	10.8	29.2	31.8	

from the mean times of those alone in which mould occurred. In only one case (no. 15) did the mould seem to have an adverse effect and in only one case (no. 14) a beneficial effect.

Long after the experiments so far described had been concluded the new yeast fructose technique, described on p. 713, was devised and new series of experiments were set up (Tables 5 and 6). In view of the large amounts of yeast present (50% of dry diet) it was not considered essential to add salts. In fact, addition of salt mixture did not alter the result if cholesterol or any other growth-promoting sterol was present. When, however, no sterols were supplied, the addition of salts usually had the effect of delaying death in a few individuals. As a consequence no salts were added in the experiments of Table 6 except where expressly stated (no. 21).

It is obvious from Table 5, nos. 16 and 17, that the activities of pure cholesterol and 7-dehydrocholesterol are about equal, although smaller quantities of the latter were used. The periods required for pupation in this series are considerably longer than in those of former experiments (Table 3). This is due mainly to the lower temperature of breeding (25 against 27°) and the lower water content (20–25 against 50%); it may also have been due in part to the lower protein content of the fructose diet and the breeding of five larvae together in one tube, instead of single cultures. On the other hand, the lower death rate in the fructose diet experiments suggests that the conditions must have been more favourable in other respects.

Of thirteen sterol preparations tested (Tables 5 and 6) only four proved to be of any nutritional value: cholesterol, dehydrocholesterol and two esters of these substances, cholesteryl acetate and 7-dehydrocholesteryl monobenzoate.

Table 5. *Yeast fructose diet; 50 mg. of sterol (10 mg. in case of dehydrocholesterol) in 3 g. of food; 25°*

No.	Sterol	No. of larvae	No. of pupae	First pupa at day	Last pupa at day	Average time of pupation days
16	Cholesterol	10	10	33	39	36
17	7-Dehydrocholesterol, 10 mg.	10	10	33	38	35.5
18	Cholesteryl acetate	10	10	32	41	36.5
19	7-Dehydrocholesteryl mono-benzoate	10	8	33	39	36

Table 6. *Yeast fructose diet; 50 mg. of sterol in 3 g. of food; 25°*

No.	Additions to diet	No. of larvae	First dead at day	Last dead at day	Average time of death days
20	No sterol, no salts added	30	16	22	19
21	No sterols, salts added	10	16	53*	24
22	Sitosterol	20	18	57*	23.4
23	Ergosterol	20	16	25	20.5
24	Zymosterol	20	16	18	16.5
25	Chloroform extract of wholemeal flour	20	16	21	18.5
26	Cholestanol	20	16	21	18.5
27	Cholestene	10	17	37*	21
				(1 pupa 53 days)	
28	7-Ketocholesteryl acetate	20	16	48*	19.8
29	7-Hydroxycholesterol	20	16	40*	18.8
30	7-Hydroxycholesteryl dibenzoate	20	16	40*	19
				(1 pupa 42 days)	

* One larva only survived for any considerable time.

The differences in the average survival times are not considered to indicate significant differences in the potencies of the inadequate diets. It was not considered advisable to disturb cultures too often, and at the first inspection usually several larvae were dead, so that the figures of the last column cannot be regarded as accurate mean values. The fact that on several diets one larva survived the others for a considerable time, and on cholestene and 7-hydroxycholesteryl dibenzoate even reached pupation, may be of some significance, but is more likely due to the presence of impurities in the sterols. This is the more probable since the chloroform-soluble fraction of whole wheat, which besides sitosterol certainly contained mixtures of other sterols and which proved to be an adequate source of sterols for several flour insects (unpublished), was entirely negative.

This also raised the question of whether some of the positive effects of sterols (Table 5) may not equally be due to impurities. The preparations which were available, with the exception of cholesterol, were admittedly not chemically pure, and it was possible that the presence of cholesterol in 7-dehydrocholesterol and in 7-dehydrocholesteryl monobenzoate might be the cause of their being of positive nutritional value. Chemically pure samples of these compounds not being obtainable, an attempt was made to determine the lowest effective concentration of each of these compounds and of pure cholesterol. Only if this concentration proved to be considerably lower in the case of cholesterol than in that of the other compounds could the presence of small quantities of cholesterol as an impurity possibly give them an apparent positive nutritional value. It was

also considered of interest to ascertain whether the presence of such large quantities of sterols as had hitherto been used was essential or whether the quantities required might be more of the order of vitamin requirements.

Accordingly, series of diets with decreasing quantities of sterols were set up (Table 7). The threshold of 7-dehydrocholesterol was found to be considerably lower than that of cholesterol. Decreasing the quantities of 7-dehydrocholesterol from 10 to 0.5 mg./3 g. of food hardly altered the efficiency of the diet, so that the threshold must be considerably lower than this. With cholesterol at quantities below 2 mg. the efficiency decreased considerably, as seen by an increasing mortality and increasing time to pupation. Since, therefore, 7-dehydrocholesterol is more effective at low concentrations than cholesterol, the possibility that cholesterol as an impurity is the cause of the positive effect given by 7-dehydrocholesterol is ruled out. The same consideration applies to 7-dehydrocholesteryl monobenzoate, the threshold of action of which does not differ greatly from that of cholesterol.

Table 7. *Yeast fructose diet; 25°*

No.	Additions to diet mg./3 g. food	No. of larvae	No. pupating	First pupa at day	Last pupa at day	Average time of pupation days
31 (=16)	Cholesterol, 50	10	10	33	39	36
32	" 10	10	10	31	41	36.5
		10	10	35	42	36.7
33	" 2	10	10	33	41	37.9
		10	10	35	41	37.3
34	" 1	10	6	35	45	40
35	" 0.5	10	2	39	49	44
36 (=17)	Dehydrocholesterol, 10	10	10	33	38	35.5
37	" 2	10	10	33	42	37.5
38	" 1	10	9	33	41	37
39	" 0.5	10	9	33	41	37
40	Dehydrocholesteryl monobenzoate, 5	10	10	34	45	38
41	" 2	10	10	38	46	41
42	" 1	10	9	38	57	47.8
43	" 0.5	10	4	45	57	50.2

The experiments of Table 7 are, of course, not real threshold experiments, because the quantity of the substance which the insects have actually eaten during development is unknown. The amount of dehydrocholesterol in the food was not further decreased, since it was not believed possible to mix such small amounts evenly into the food.

DISCUSSION

The only previous attempt to compare the roles of several sterols as food factors was undertaken by Van t'Hoog [1936] on *Drosophila*. *Drosophila* larvae were found to grow well on a sterile diet consisting of peptone from purified casein, sugar, a collodion dialysate of yeast extract, salts, agar-agar and water, if to this food was added a small quantity of the unsaponifiable fraction of fat [Van t'Hoog, 1935]. Later, Van t'Hoog [1936] found that this unsaponifiable fraction could be replaced successfully by a number of sterols: cholesterol (purified via the dibromide), sitosterol, stigmasterol, ergosterol, cholestanol (dihydrocholesterol) and cholestenone. Calciferol, on the contrary, and several derivatives of cholesterol, proved to be ineffective. The flour beetle *Tribolium confusum* also requires in its diet either cholesterol or ergosterol [Fröbrich, 1939; Offhaus, 1939]. The differences in the sterol requirements of two different insects, *Drosophila* and *Dermestes*, are remarkable. Cholesterol, sitosterol and stigmasterol

possess the same ring system with only one double bond in ring B and differ solely in the side chain on carbon atom 17. This difference is of no importance for *Drosophila* and of great importance for *Dermestes*, which cannot develop on sitosterol. Ergosterol and 7-dehydrocholesterol contain a second double bond in ring B and differ from each other only in the side chain. While ergosterol is active for *Drosophila* and *Tribolium* it is entirely inactive for *Dermestes*. This negative effect on *Dermestes* is obviously due to the side chain and not to the second double bond, because 7-dehydrocholesterol, which possesses the ring system of ergosterol and the side chain of cholesterol, is active for *Dermestes*. Cholestanol, which differs from cholesterol only in the absence of the double bond in ring B, is positive for *Drosophila* and negative for *Dermestes*, which indicates that the presence and position of the double bond is of importance too. Zymosterol, with one double bond in a different place in ring C and a side chain which is different from that of cholesterol, is also inactive.

Of the derivatives of cholesterol and dehydrocholesterol tested, only the esters of these compounds, cholesteryl acetate and 7-dehydrocholesteryl monobenzoate, have a positive effect, which suggests that hydrolysis of these esters takes place in the larva. Other changes in the configuration, such as the loss of the double bond in cholestanol, loss of the secondary alcohol group in Δ^5 -cholestene, introduction of a second alcohol group in 7-hydroxycholesterol and esterification of this group in 7-hydroxycholesteryl dibenzoate or introduction of a keto-group on carbon atom 7 in 7-ketocholesteryl acetate, cause loss of nutritional value. It therefore appears, as far as can be judged from the number of compounds available for testing, that only substances with the configuration of cholesterol and dehydrocholesterol or their esters can be utilized by *Dermestes*. Change in the side chain, the number and position of double bonds, opening out of one ring, introduction of a second alcohol or a keto-group destroy the effect entirely.

The differences in the requirements for sterols in *Drosophila* and *Dermestes* are interesting. *Dermestes* feeds exclusively on relatively dry products of animal origin like stored hides, bacon, smoked meat, dried-up cadavers and the like in which cholesterol constitutes the only or main sterol present. *Drosophila* develops on moist food rich in carbohydrates in which yeast grows abundantly. In fact, it is generally considered that living yeast is the main food of the *Drosophila* larva [Guyénot, 1917]. It is therefore illuminating to find that the sterol requirements of *Drosophila* are fulfilled by ergosterol, the principal sterol of yeast, whereas as far as we know to-day cholesterol and 7-dehydrocholesterol, which appears to be generally associated with cholesterol, constitute the only active sterols for *Dermestes*. It will be shown in a later publication that the requirements of several flour insects for sterols are, amongst other sterols, met by sitosterol, the principal sterol of wheat germ oil.

In recent years the antirachitic effect of irradiated cholesterol on rats has been shown to be due to the presence before irradiation of 7-dehydrocholesterol as an impurity. It is, however, certain that for both *Drosophila* and *Dermestes* the presence of this impurity is immaterial, for purified cholesterol proved as effective as commercial cholesterol. The insects, then, require in their food a sterol, and it is not surprising that this requirement is not fulfilled by calciferol, in which the tetracyclic arrangement of the carbon atoms has been changed, and which is consequently not a true sterol.

So far it has been shown for at least five different insects, namely two flies, *Lucilia* [Hobson, 1935] and *Drosophila* [Van t'Hoog, 1936], three beetles, *Acanthoscelides* [Chiu & McCay, 1939], *Tribolium* [Fröbrich, 1939; Offhaus, 1939]

and *Dermestes*, and for a snail *Helix pomatia* [Howes, 1937], that the inclusion of a sterol in the diet is essential for growth. This also applies to four more beetles: *Lasioderma*, *Sitodrepa*, *Silvanus* and *Ptinus*, and one moth *Ephestia* [Fraenkel, unpublished]. It seems, however, that the requirements of living organisms for sterols are of a more general occurrence than has hitherto been supposed. Cailleau [1938] has shown that the parasitic flagellates *Trichomonas columbae* and *Tr. foetus* only grow in the presence of cholesterol. A certain strain (*W*) of yeast requires for growth a sterol in addition to bios; cholesterol is inactive but sitosterol, ergosterol and calciferol are active [Devloo, 1938]. The position therefore is that invertebrates and even some micro-organisms require sterols but do not require the irradiated products of sterols (vitamin D). In the light of these findings it seems that the almost universally accepted thesis of the synthesis of cholesterol in the mammalian body might require revision. Recent evidence on this subject is conflicting [Channon & Tristram, 1937; Rittenberg & Schoenheimer, 1937], and it has only recently been pointed out by Bacharach & Drummond [1940] that the question whether mammals are able to synthesize cholesterol is entirely unsettled.

SUMMARY

1. Several methods for investigating the sterol requirements of the beetle *Dermestes vulpinus* have been described.

2. The larva of *D. vulpinus* only grows if the food contains a sterol. Active sterols include purified cholesterol (free from 7-dehydrocholesterol), cholesteryl acetate, 7-dehydrocholesterol and 7-dehydrocholesteryl monobenzoate. Sitosterol, ergosterol, zymosterol, cholestanol, cholestene, 7-ketocholesteryl acetate, 7-hydroxycholesterol, 7-hydroxycholesteryl dibenzoate and calciferol (vitamin D₂) are inactive.

3. The minimum quantity of sterol required for successful growth is about 1 mg./3 g. of food for cholesterol and 7-dehydrocholesteryl monobenzoate and less than 0.5 mg./3 g. of food for 7-dehydrocholesterol.

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