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The Nutrition of the Larva of *Aedes aegypti* Linnaeus

3. LIPID REQUIREMENTS

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In a previous communication (Golberg, De Meillon & Lavoipierre, 1945) we showed that water-extracted yeast residue, supplemented by a number of water-soluble vitamins, constitutes an adequate medium for the growth and metamorphosis of the larva of *Aedes aegypti* Linnaeus. From this starting point our investigations were concerned with the part played by lipids in the nutrition of the mosquito larva.

Considering the ability of insects to transform carbohydrate and protein into fat (evidence summarized by Wigglesworth, 1942), it is surprising to find that many insects are incapable of synthesizing sterols; or at least of producing them in sufficient quantities to cover their needs for growth and particularly for metamorphosis. The need for pre-formed sterol in the diet of many insects has been conclusively demonstrated. Yet serious consideration does not appear to have been given to the possibility that non-steroid lipids may be capable of fulfilling the same nutritive purpose. For example, iecithin has been employed as a component of diets for *Drosophila* (Bacot & Harden, 1922; Guyénot, 1917; Lafon, 1937), and frequent reference is made in the literature to essential lipid factors present in wheat, wheat germ, cod-liver oil, butter fat, and ethanolic extract of yeast. In two instances, in which

attempts have been made to fractionate the fat and isolate the active agent, the conclusion was reached that cholesterol was the essential constituent (Hobson, 1935; van't Hoog, 1936). Fraenkel & Blewett (1945) found that, in addition to cholesterol, insects of the genus *Ephestia* require linoleic acid and vitamin E.

Gay (1938) and subsequent workers (Fraenkel, Reid & Blewett, 1941; Fraenkel & Blewett, 1943) employed yeast in the growth media used for studying the sterol requirements of insects. Although the proportion of yeast in the dietary solids varied from 5 up to 96%, they, nevertheless, found it necessary to add a further quantity of cholesterol to the diet, the minimum being 1 mg. sterol/3 g. diet (Fraenkel *et al.* 1941). Dried yeast has a fat content of 7% (Newman & Anderson, 1933) of which approximately one third consists of sterols (Smedley-MacLean & Thomas, 1920). It would appear that this level of sterols is inadequate for insects normally feeding on flour or other stored food, particularly when the yeast constitutes only 5% of the artificial diet. In the case of *Aedes* larvae, however, when the whole of the solid portion of the growth medium was composed of yeast, no added sterols or other lipids were needed.

EXPERIMENTAL

Aedes aegypti L. The strain of mosquitoes used was the same as in our previous investigations (De Meillon, Golberg & Lavoipierre, 1945; Golberg *et al.* 1945).

Method of obtaining sterile eggs and larvae of Aedes aegypti. In order to ensure that the eggs used were not more than 24 hr. old, the following procedure was employed: gravid females from the insectary were confined, singly, in sterile test tubes, together with 2-3 ml. sterile distilled water and incubated at 28°. The tubes were examined every morning and the females liberated from those tubes containing eggs. The eggs and water were poured with aseptic precautions into a fresh sterile tube, and the water withdrawn.

Tubes with stranded eggs were now half-filled with White's solution (White, 1931), left for 30 min., the fluid removed and the eggs washed three times with sterile distilled water. In the final washing a platinum loop was used to dislodge any eggs adhering to the sides of the tube. The sterilized eggs were transferred directly to tubes containing an autoclaved solution of glucose (1%) and Tatum's (1939) salt mixture (0.15%), previously adjusted to pH 6.5. Incubation at 28° was continued for 96 hr. after the larvae had hatched, and served to detect contaminated batches. Those found to be sterile were used forthwith.

Preparation of media. As a rule each experimental medium consisted of a liquid and a solid component, prepared and sterilized separately as follows: *The liquid component* was invariably prepared fresh and sterilized on the same day by pressure filtration through a pad. The sterile filtrate was collected in a special apparatus designed to facilitate rapid distribution of the liquid into tubes containing the sterile solid fraction. In general 10 ml. were measured into each tube. Where sodium caseinate was used, it was autoclaved and added separately. *The solid component* was sterilized by autoclaving for 30 min. at 15 lb. pressure. A quantity of 20-50 mg. per tube was adequate, but observation of the larva was facilitated by adding an amount sufficient to fill the rounded portion of the tube. Lipids were introduced into the solid fraction of the medium by solution in a small volume of pure ether (or other suitable volatile solvent) and stirring with powdered cellulose or kaolin until the solvent had evaporated. Such mixtures were autoclaved in the dry state.

Media were tested for sterility in the usual manner.

Observations of development and methods of recording them. The stage of development reached by each larva was recorded every morning and evening, and the time taken for the change from one stage to the next was calculated to the nearest half-day. In considering the results of experiments, it was thus possible to compare the times taken for each stage ('growth rates') and also the number of larvae reaching that stage ('survival rates'). It is realized that the growth rates recorded are subject to a maximum error of +0.5 day, but this does not affect the standard deviations or the significance of differences between groups.

In most tables the results have been condensed, but all the data are available in the same detail as in Tables 1 and 2.

Materials and composition of media

The basal medium (LM) contained glucose (10 g./l.), salt mixture (Tatum, 1939) (1.5 g./l.) and the following vitamins (in mg./l.): aneurin chloride, 10; riboflavin, 20; pyridoxin hydrochloride, 10; calcium D-pantothenate, 10; nicotinic acid, 10. The pH was adjusted to 6.3-6.5.

For use with sodium caseinate the basal medium was prepared in double strength and 5 ml. filtered medium added to 5 ml. autoclaved sodium caseinate.

Sodium caseinate was at first prepared from purified casein. Later, S.M.A. Co. 'Vitamin test' casein was used, and the pH adjusted to 6.6. Immediate autoclaving at 18 lb. for 45 min. was essential to prevent contamination. Analysis showed the average concentration of sodium caseinate in the final medium to be 0.95% (w/v).

Yeast autolysate. Difco 'Bacto yeast extract' was used in a concentration of 2% (w/v).

Yeast residues were prepared from fresh brewer's yeast which was dehydrated with ethanol, extracted at room temperature for 24 hr. with ethanol, followed by a mixture of ethanol and light petroleum (1:1). After five additional extractions with boiling mixtures of ethanol and benzene (2:1) (Bills, Massengale & Prickett, 1930) it was dried in air, powdered and sieved.

Newman & Anderson (1933) showed that treatment with ethanolic hydrogen chloride was necessary for the extraction of a small quantity of yeast fat which cannot be removed by neutral solvents. Accordingly, the yeast residue as prepared above was incubated for 2 days at 37° with 1% (w/w) ethanolic hydrogen chloride, after which it was washed and dried as before.

Yeast or yeast residue was freed from water-soluble constituents by repeated extraction with boiling water and finally by continuous extraction with boiling water in a Soxhlet apparatus for 48 hr.

Lipids. Freshly prepared and purified egg-yolk lecithin and beef-brain cephalin were submitted to additional purification as described by Weil-Malherbe & Dickens (1944). Careful testing failed to reveal the presence of sterols in the purified materials. Sphingomyelin (beef brain) was purified by the procedure of Levene (1916). Kerasin was prepared from the fresh spleen of a case of Gaucher's disease, supplied by Dr J. A. Murray of this Institute.

Steroids. Many of the sterols and derivatives used were prepared by standard procedures. The remainder, including sex hormones and related compounds, were obtained from Drs F. W. Fox and J. Gillman, Prof. I. M. Heilbron and Dr E. R. H. Jones, Mr F. A. Robinson (Glaxo Laboratories Ltd.), Profs. L. Ruzicka and Pl. A. Plattner, Dr E. Schwenk (Research Division, Schering Corporation) and Dr H. A. Shapiro.

All steroids used were checked for purity and where necessary submitted to further purification. Only in the case of 7-dehydrocholesterol was the test material of doubtful purity.

Methyl linoleate and linolenate were obtained from Prof. T. P. Hilditch.

Folic acid. A concentrate of the ammonium salt of folic acid, of potency 5000, obtained from Prof. R. J. Williams, was used in a concentration of 20 mg./l. of medium.

Pteroylglutamic acid was made available to us by Dr E. L. R. Stokstad (Lederle Laboratories). It was used in a concentration of 25 mg./l.

RESULTS

The growth-promoting properties of fat-free yeast and yeast lipids

The basal medium (*LM*) containing glucose, salts, aneurin, riboflavin, pyridoxin, nicotinic and pantothenic acids, when combined with fresh brewer's yeast, produced excellent growth of the larvae. Gradual removal of the fats from the yeast decreased the rate of growth and the number of pupae and adult mosquitoes. Finally, after extraction of the yeast residue with ethanolic hydrogen chloride, there was complete failure to emerge from the pupal stage (Table 1). Recombination of the final yeast residue with the yeast lipids led to normal growth and survival rates, provided that the lipid concentration did not exceed 4%. At 8% lipid concentration the medium was very toxic.

Further experiments were designed to supplement fat-free yeast residue with yeast lipid fractions and with a variety of lipids from other sources. The crude yeast fat was able to produce normal growth. Its ether-soluble fraction did the same. Of the other fractions tested, that obtained by steam-distilling yeast fat was remarkably toxic. In a concentration of 0.15% on the solid component of the medium it completely prevented growth of the larvae. At higher concentrations it killed them within 24 hr.

Admixture of fat-free yeast residue with cholesterol, purified ovolcithin or beef-brain kephalin in each case resulted in growth to the adult stage. The best results were obtained by the use of all three lipids in a total concentration of 2.5%, resulting in growth and survival rates closely similar to those observed with yeast fat.

Table 1. *Rates of growth and survival of mosquito larvae on media containing fat-free yeast*

(Here and in subsequent tables: No.=number in stage of development indicated. The number of first-instar larvae taken was approximately 20 per experiment. Mean; s.d.=mean and standard deviation of time (in days) taken to change from previous stage of development.)

Medium	Larval instars						Pupae				Adults	
	II		III		IV		Mean;		Mean;		Total time taken	
	No.	Mean; s.d.	No.	Mean; s.d.	No.	Mean; s.d.	No.	s.d.	No.	s.d.	No.	Range Mean; s.d.
<i>LM</i> , with additions as indicated:												
Fresh brewer's yeast	63	1.9±0.36	63	1.1±0.19	63	1.1±0.29	60	2.6±0.50	57	—	7.5-11.5	8.8±0.60
Fat-free yeast residue	48	2.6±0.48	48	2.1±0.57	48	3.2±1.03	17	10.7±2.36	0	—	—	—
Fat-free yeast + yeast lipids (2.5%)	23	2.2±0.29	22	1.3±0.41	22	1.6±0.38	20	3.0±0.62	18	—	8.5-9.5	9.1±0.49
Water-extracted fat-free yeast	7	4.1±1.60	7	2.9±0.83	0	—	—	—	—	—	—	—
Water-extracted fat-free yeast + folic acid	18	1.9±0.99	16	1.9±0.75	1	3.0	—	—	—	—	—	—
Fat-free water-extracted yeast	22	3.8±0.86	20	5.0±1.65	11	5.4±1.09	0	—	—	—	—	—
Fat-free water-extracted yeast + pteroylglutamic acid	21	1.9±0.63	21	1.6±0.84	12	3.8±3.61	2	5.8	0	—	—	—
Fat-free water-extracted yeast + yeast autolysate	17	5.0±3.08	16	1.4±0.45	14	3.2±1.96	1	15.0	0	—	—	—
Fat-free yeast + cholesterol	20	2.7±0.92	20	1.4±0.54	20	1.7±0.55	17	3.9±0.62	16	—	9.5-15.5	11.6±1.60
Fat-free yeast + lecithin	19	2.8±0.88	19	1.9±0.93	17	2.5±1.32	16	3.8±0.50	16	—	9.5-16.5	12.6±2.10
Fat-free yeast + kephalin	22	2.9±0.98	20	2.3±0.67	20	2.8±1.48	14	5.5±1.44	12	—	11.5-20	15.1±2.73
Fat-free yeast + cholesterol, lecithin and kephalin (1:1:1)	19	1.6±0.16	19	1.2±0.31	19	1.3±0.49	19	3.4±0.55	19	—	8.5-10.5	9.7±0.85

Exhaustive extraction of fat-free yeast with water rendered it incapable of supporting growth, even to the fourth larval instar. The addition of folic acid to the medium made no essential difference. Reversal of the order of extraction, using water first and fat solvents later, yielded a residue which promoted better growth and afforded clear evidence of the effect of lipid deficiency on growth and survival of mosquito larvae (Table 1).

Growth on other fat-free media

A study was made of the growth of larvae on fat-free media not containing solid yeast. Casein, as the source of protein, gave best results when present in solution as the sodium salt. By incorporating yeast autolysate in the medium it was found that the fat-free yeast residue could be dispensed with (Table 2). In none of our experimental media could a need for

solid material be demonstrated. However, it was convenient to use a solid in finely divided form as a means of introducing lipids into the medium; and for this purpose cellulose, cellulose acetate or kaolin were equally effective.

Fatty acids and triglycerides

Since a number of compounds seemed capable of bringing about the lipid effect, it was deemed advisable to make sure that this property did not

Table 2. Rate of growth and survival of mosquito larvae on media not containing fat-free yeast residue*

Medium	Larval instars						Adults				
	II		III		IV		Pupae		Total time taken		
	No.	Mean; S.D.	No.	Mean; S.D.	No.	Mean; S.D.	No.	Mean; S.D.	No.	Range	Mean; S.D.
<i>LM</i> + yeast autolysate, with additions as indicated:											
Casein	36	2.7 ± 1.41	35	2.2 ± 0.78	16	5.1 ± 4.37	0	—	—	—	—
Sodium caseinate	20	3.1 ± 0.33	20	1.4 ± 0.39	18	2.4 ± 1.42	8	5.9 ± 1.19	0	—	—
Sodium caseinate + cellulose	21	1.9 ± 0.37	20	1.6 ± 0.21	16	3.0 ± 1.44	3	7.0	1	—	12.5
Sodium caseinate + kaolin	34	1.8 ± 0.63	34	1.9 ± 0.48	25	3.2 ± 1.85	7	6.5 ± 0.76	3	12.5–15	14.0
<i>LM</i> + yeast autolysate + sodium caseinate + lipids in 2.5 % concentration on cellulose:											
Cholesterol	17	2.0 ± 0.51	17	1.3 ± 0.24	17	1.8 ± 0.25	13	3.8 ± 0.75	9	9.5–12.5	11.0 ± 1.04
Lecithin	24	1.8 ± 0.38	24	1.4 ± 0.36	24	1.9 ± 0.39	22	4.4 ± 1.14	16	9.5–14.5	11.9 ± 1.49
Kephalin	25	1.7 ± 0.32	25	1.6 ± 0.42	24	2.4 ± 1.36	8	5.3 ± 2.10	1	—	10.0
Cholesterol + lecithin + kephalin (1 : 2 : 1)	40	2.1 ± 0.67	40	1.5 ± 0.42	39	1.9 ± 0.51	34	3.4 ± 0.65	32	9.5–13	10.8 ± 1.09
Sphingomyelin	17	2.4 ± 0.78	17	1.2 ± 0.60	17	1.7 ± 0.69	11	3.9 ± 0.63	7	9.5–11	10.5 ± 0.60
Kerasin	22	2.3 ± 0.89	22	1.3 ± 0.42	22	1.8 ± 0.38	18	3.8 ± 0.65	16	10.5–13	11.4 ± 0.92
β-Carotene	44	3.3 ± 0.76	44	2.0 ± 0.60	44	2.8 ± 1.48	31	6.2 ± 2.10	23	11 –29	16.9 ± 2.81
α-Tocopherol	37	2.3 ± 0.39	37	2.2 ± 0.61	30	3.4 ± 0.81	15	8.3 ± 2.48	11	13 –23.5	16.8 ± 2.11
α-Tocopheryl acetate	15	2.4 ± 0.60	15	2.0 ± 0.45	15	2.7 ± 0.69	12	8.4 ± 2.63	8	12.5–24.5	17.5 ± 3.08

* For explanation of headings see Table 1.

Table 3. The effect of cholesteryl esters on growth and metamorphosis of mosquito larvae

(Medium: *LM* + sodium caseinate + yeast autolysate + cholesteryl ester (2.5%) on cellulose.)

Cholesteryl ester tested	No. of second larval instars	No. of adult mosquitoes	Total time taken	
			Mean	S.D.
Unesterified	22	11	11.8 ± 0.97	
Acetate	23	14	10.6 ± 0.69	
Palmitate	17	12	13.5 ± 1.74	
Stearate	20	11	13.4 ± 2.92	
Oleate	14	5	13.3 ± 1.29	
Cerotate	18	6	13.7 ± 1.32	
Arachidate	17	9	16.9 ± 4.36	
p-Aminobenzoate	17	15	18.0 ± 1.90	
Nicotinate	19	15	12.4 ± 2.37	

A medium was thus available for testing the effects of various lipids. Cholesterol and lecithin were capable of bringing about pupation of the larvae and emergence of adults, but cephalin was totally ineffective. On combining these lipids in pairs, or using all three in various proportions, the rates of growth were but little affected, while the rates of survival varied considerably. The highest proportion of adult mosquitoes (80%) resulted from the use of lecithin, cholesterol and kephalin in the proportion of 2:1:1. This mixture was adopted as the standard source of lipids for subsequent 'synthetic' growth media.

Of the other lipids tested under the same conditions, only kersin and, to a lesser extent, sphingomyelin, β-carotene and α-tocopheryl acetate, were capable of replacing cholesterol.

extend to the common fatty acids and triglycerides. None of the following could replace cholesterol: palmitic, stearic and oleic acids; tripalmitin, tristearin and triolein. Lauric and myristic acids were highly toxic; so too was oleic acid, even in a concentration of 0.5% on kaolin. More highly unsaturated fatty acids—linoleic and linolenic acids, even when used with α-tocopherol (Fraenkel & Blewett, 1945) were inactive. Furthermore, all the above-mentioned compounds proved incapable of supplementing cholesterol in the way in which lecithin and kephalin had done. Similarly, esterification of cholesterol with acetic, oleic, palmitic, stearic, cerotic and arachidic acids had no striking beneficial effect on larval growth and survival (Table 3).

Lipids in solution

On testing the effect of presenting the lipids in solution, it was found that neither lecithin nor cholesterol had any apparent effect. Nor was lecithin in solution able to improve the larval response to cholesterol present in the solid state (Table 4). Fatty acids in solution as the sodium salts were tested under the same conditions: palmitate and stearate had no effect and oleate displayed no toxicity. The concentrations tested were of necessity rather low (0.25 %).

results given in Table 5 indicate considerable variation in this respect. In fact, the principal sterols of yeast, when provided in pure form, appear incapable of utilization by the mosquito larva. However, under the conditions of these experiments no sterol—not even cholesterol—produced a wholly satisfactory response, particularly with regard to the proportion of larvae reaching the adult stage. In order to render the medium more fully adequate for the promotion of optimal growth and survival, the powdered cellulose was replaced by a water-extracted yeast residue from which all lipids soluble in neutral

Table 4. *The effect of lipids in solution on growth and metamorphosis of mosquito larvae*

(Medium: LM + sodium caseinate + yeast autolysate.)

Lipid in solution	Concentration (g./l.)	Lipid on cellulose (4% concentration)	No. of second larval instars	No. of adult mosquitoes	Total time taken	
					Mean	S.D.
None	—	None	28	2	13.0	
Lecithin	0.4	None	69	5	14.5	
Lecithin	0.25	None	15	2	13.0	
Lecithin	0.4	Cholesterol	23	10	10.9 ± 0.87	
Lecithin	0.25	Cholesterol + kephalin (1 : 1)	63	38	12.5 ± 0.99	
Lecithin	0.4	Kephalin	18	1	10.5	
None	—	Lecithin	24	16	11.9 ± 1.49	
Cholesterol	0.05	None	23	1	18.5	

Table 5. *The effects of various sterols on growth and metamorphosis of mosquito larvae*

(Medium: LM + sodium caseinate + yeast autolysate + sterol (2.5 %) on cellulose.)

Sterol tested	No. of second instar larvae	No. of adult mosquitoes	Total time taken	
			Mean	S.D.
Cholesterol	22	11	11.8 ± 0.97	
Cholesterol (purified through the dibromide)	39	15	11.5 ± 1.53	
Coprosterol	22	10	13.5 ± 1.87	
β-Sitosterol	43	32	10.7 ± 1.12	
Stigmasterol (acetate)	23	10	10.6 ± 0.59	
Ergosterol	21	7	9.2 ± 0.84	
Zymosterol	21	1	10.0	

Table 6. *The ability of various sterols to replace cholesterol in a medium containing fat-free water-extracted yeast residue*

(Medium: LM + sodium caseinate + yeast autolysate + sterol (2.5 %) on fat-free water-extracted yeast.)

Sterol tested	No. of second instar larvae	No. of adult mosquitoes	Total time taken	
			Mean	S.D.
Cholesterol	20	19	9.4 ± 0.96	
Coprosterol	19	19	12.5 ± 1.80	
β-Sitosterol	15	14	9.5 ± 1.30	
Stigmasterol (acetate)	20	20	8.8 ± 0.73	
Ergosterol	18	12	9.4 ± 0.84	
Zymosterol	19	16	14.1 ± 2.56	
None	17	0	—	

The ability of other sterols to replace cholesterol in the larval diet

It is most unlikely that cholesterol is normally available to the mosquito larva in its natural habitat. Hence the question arises: to what extent can other sterols replace cholesterol in the larval diet? The

organic solvents had been removed. This preparation still contained a small amount of lipid extractable by incubation with ethanolic hydrogen chloride. The residual lipid did not prevent a complete failure to pupate in the absence of added sterol; yet it ensured that when cholesterol was added excellent growth and survival resulted (Table 6). Under these

conditions the plant sterols were fully equal to cholesterol in their effects. The mycosterols were definitely inferior to the others, zymosterol producing a poor rate of growth, ergosterol a low proportion of adults.

Modifications of sterol structure

These results made it desirable to inquire into the effects of modifications in the sterol structure, and to investigate the relationship of the activity of such sterol derivatives to other dietary factors. For this purpose each compound was tested on three solid components in the usual liquid medium. The solids used were: cellulose, fat-free water-extracted yeast, and the yeast residue prior to extraction with ethanolic hydrogen chloride. The conclusions to be drawn from the results, some of which are given in Table 7, may be summarized thus: (a) none of the

pounds of the pregnane, androstane and oestrane series: Δ^5 -pregnen-3-ol-20-one; dehydroisoandrosterone acetate; progesterone; deoxycorticosterone acetate; 17-ethinyl- and 17-methyl-testosterone; testosterone itself and its propionate; Δ^4 -androstene-3:17-dione; pregnane-3(α):20 α -diol; *allopregnane*-3(α):20 α -diol; androsterone; *allopregnane*-3:20-dione; oestrone and oestradiol benzoate; (d) among the inactive compounds were some which were markedly growth-inhibitory (marked with an asterisk above).

DISCUSSION

In the experiments described above, the progress of each individual larva was recorded. In consequence it was possible to demonstrate that a lipid-free medium supports larval growth, albeit at a somewhat reduced rate, to the fourth instar. The effect of lipid deficiency reveals itself most frequently in a failure

Table 7. *The growth-promoting properties of sterol derivatives*

(Media: LM + sodium caseinate + yeast autolysate + test compound (2.5%) on: A, cellulose; B, yeast extracted with neutral organic solvents and water; C, yeast B further extracted with ethanolic hydrogen chloride.)

Sterol derivatives tested	No. of second instar larvae	No. of fourth instar larvae	No. of adult mosquitoes	Total time taken	
				Mean	S.D.
β -Cholestanol on A	22	14	6	12.4	± 0.61
B	18	17	12	12.8	± 1.04
C	17	17	12	12.5	± 1.77
Cholesterol dibromide on A	19	19	9	11.7	± 1.05
B	22	22	21	13.0	± 2.86
C	20	19	19	12.3	± 1.31
3(β)-Hydroxy-6-ketocholestane on A	16	12	0	—	—
B	18	17	9	13.6	± 2.07
C	18	16	7	14.9	± 1.82
Δ^4 -3(β):6-Dihydroxycholestene on A	21	13	0	—	—
B	22	22	20	12.1	± 1.58
C	19	17	14	12.8	± 1.93
7-Dehydrocholesterol on A	21	18	1	12.0	—
B	17	17	14	12.5	± 1.60
C	18	18	17	11.8	± 1.27

compounds tested showed any striking degree of activity on cellulose; most were completely inactive; (b) in the presence of both types of yeast residue the following compounds displayed activity: β -cholestanol, cholesterol dibromide, Δ^4 -3(β):6-dihydroxycholestene, 3(β)-hydroxy-6-ketocholestane. There was remarkable correspondence between the growth and survival rates on the two types of yeast residue; (c) all other compounds tested proved inactive. These included the following cholesterol derivatives: Δ^4 -cholestenone* and its enol acetate*, α -7-hydroxy- and 7-ketocholesterols, $\Delta^{4,6}$ -3-ketocholestadiene*, $\Delta^{3,5}$ -7-ketocholestadiene* 3(β):5:6-(*trans*)trihydroxycholestane*, *cis*- and *trans*- Δ^5 -cholestene-3:4-diols and their diacetates, and methyl Δ^5 -3-acetoxy-bisnorcholesterol. Calciferol was inactive, as well as the following steroid hormones and related com-

ounds to undergo metamorphosis. In those few instances in which pupation does occur, it takes place after a considerable delay, and only a small proportion of adults emerge.

Lipid deficiency can be corrected, but not completely, by cholesterol or lecithin, kerosin and, to a lesser degree, by other lipids. In addition, the action of lipids can be supplementary, and a mixture of cholesterol, lecithin and cephalin gives better results than either cholesterol or lecithin alone (cephalin alone is inactive). There are thus two aspects of lipid action to be considered, which may be referred to briefly as replacement and supplementation of cholesterol.

The ability of lecithin to replace cholesterol cannot be attributed to the presence in it of sterol as an impurity (see p. 373). Also the activity of lecithin

does not appear to be due to component unsaturated fatty acids, since such fatty acids proved incapable of replacing or supplementing cholesterol. This, taken in conjunction with the fact that relatively few steroid derivatives evince this type of activity, leads to the conclusion that the specialized nutritive power is a function of special types of molecules. It is impossible to state at this stage what these molecules have in common. There is not a physicochemical influence on the external medium of the mosquito larva since, when present in solution in the medium, they are inactive. On the other hand, there is a definite parallelism between their effects on the mosquito larva and their ability to overcome the growth inhibition of Gram-positive bacteria induced by linoleic acid. This effect Kodicek & Worden (1945) have attributed to surface activity.

Supplementation of cholesterol is brought about most effectively by fat-free yeast residue. However, attempts to isolate a specific supplementary factor from the yeast residue have failed. One is reminded of the animal dietary factor found necessary by Dorée & Gardner (1938) and Rosenheim & Webster (1941) for the conversion of cholesterol to coprosterol and for analogous metabolic changes. It is suggestive too that some cholesterol derivatives which are inactive when tested alone evince a measure of activity in the presence of the yeast factor.

While some sterols are capable of replacing cholesterol in the diet of the mosquito larva, on the whole little modification of sterol structure is tolerated. This statement is borne out by the findings of other workers who have studied the ability of insects to utilize sterols and cholesterol derivatives (Fraenkel *et al.* 1941; Fraenkel & Blewett, 1943; van't Hoog, 1936). The paucity of the information available makes it all the more regrettable that Fraenkel & Blewett (1943) used impure cholesterol derivatives in their investigations. The most exhaustive study of this type was made by Cailleau (1937) on the ability of sterols to act as growth factors for *Trichomonas columbae*. Although her results are not necessarily applicable to insects, it is interesting that in most instances the ability of the flagellate to utilize sterols, and particularly cholesterol derivatives, runs strictly parallel to that of the mosquito larva. The outstanding differences lie in coprosterol and stigmasterol, which Cailleau found to be inactive.

Finally, it had been hoped by means of these studies to throw some light on the possible metabolism of cholesterol in the mosquito larva. Δ^4 -3:6-Dihydroxycholestene and 3-hydroxy-6-ketocholestane are the only two cholesterol transformation products which are active for the mosquito larva,

and at the same time may be regarded as likely to occur in normal cells during life (Haslewood, 1944). Even these are active only in the presence of the yeast factor. The conversion of cholesterol to coprosterol takes place through the intermediate stage of Δ^4 -cholestenone (Schoenheimer, Rittenberg & Graff, 1935; Anchel & Schoenheimer, 1938; Marker, Wittbecker, Wagner & Turner, 1942). Turfitt (1944, 1946) demonstrated the microbiological conversion of cholesterol to Δ^4 -cholestenone. In the mosquito larva Δ^4 -cholestenone is not only inactive but also strongly inhibitory; conversion to the enol acetate does not abolish the inhibitory effect. In keeping with these observations is the lack of activity of *cis*- Δ^5 -cholestene-3:4-diol, which Rosenheim & Starling (1937) found to be readily dehydrated to cholestenone by mild acid treatment.

SUMMARY

1. A study has been made of the part played by lipids in the nutrition of the mosquito larva. The lipids were mixed intimately with an inert solid such as cellulose or kaolin or with fat-free yeast residue and introduced into a basal liquid medium.
2. It was found that in a fat-free medium the rates of growth and survival of the mosquito larva do not fall far short of normal. There are, however, few pupae and very few adult mosquitoes.
3. The larval need for lipid may be supplied by cholesterol or ovolecithin and, less successfully, by a number of other lipids. Of all those tested, no single compound, and no cholesterol ester, was capable of producing normal growth and survival unless fat-free yeast residue was included in the growth medium. Mixtures of lipids were more effective.
4. The following compounds proved inactive: saturated and unsaturated fatty acids; triglycerides; lecithin and cholesterol *in solution* in the growth medium.
5. A number of steroids, including cholesterol derivatives, were tested in the presence or absence of fat-free yeast residue. All but four were inactive.
6. The need for special lipids appears to be common to many insects and lower organisms and to be capable of being satisfied only by a limited number of compounds. The possibility exists that the effect exerted by these compounds is physicochemical rather than nutritional.

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The Nutrition of the Larva of *Aedes aegypti* Linnaeus

4. PROTEIN AND AMINO-ACID REQUIREMENTS

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The state of confusion which exists in the literature on the protein and amino-acid requirements of insects may be attributed to two causes: failure to maintain strictly sterile conditions and failure to ensure adequacy of the medium with respect to all factors other than protein. In turning our attention to the mosquito larva's needs for protein and amino-acids we have sought to avoid both pitfalls and can claim to have succeeded with regard to the first. As regards the second, our efforts have not yet culminated in a medium composed of purified individual chemical substances which could produce growth equal to that attained with the original fresh brewer's yeast medium (De Meillon, Golberg & Lavoipierre, 1945). Until that stage is reached, a final answer to some of the questions raised in this paper cannot be given. Meanwhile, it should be emphasized that growth on the media used fell short of what had been produced in the presence of yeast residue. Not only were the growth and survival rates somewhat poorer, but it was obvious that the spontaneity and uniformity which had characterized the response to whole yeast was lacking.

EXPERIMENTAL

Methods. The methods used were the same as in our previous investigations (Golberg & De Meillon, 1947). The microbiological estimation of L-valine was carried out by the method of McMahan & Snell (1944).

Media. The basal medium (CM) consisted of two components: (i) a solid component made up of 0.25 g. of a lipid mixture (lecithin, cholesterol and kephalin in the proportions 2:1:1) intimately mixed with 10 g. of finely powdered cellulose, and sterilized by autoclaving for 30 min. at 15 lb. pressure; and (ii) a liquid component made up of two halves: one half, which was sterilized by filtration through a pad, contained glucose (10 g./l.), yeast autolysate (20 g./l.), Tatum's (1941) salt mixture (1.5 g./l.) and vitamins (aneurin chloride, 10 mg./l.; riboflavin, 20 mg./l.; pyridoxin hydrochloride, 10 mg./l.; calcium D-pantothenate, 10 mg./l.; and nicotinic acid, 10 mg./l.). The other half of the liquid component, which was sterilized by autoclaving for 45 min. at 18 lb. pressure, contained the protein in solution.

Amino-acid mixtures were added to the first half of the liquid component prior to filtration. In all instances sterilization of the medium followed its preparation with the shortest possible interval for it was found that even slight contamination incidental to keeping a protein or amino-acid