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The Nutrition of the Larva of *Aëdes aegypti* Linnaeus

4. PROTEIN AND AMINO-ACID REQUIREMENTS

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(*Received* 17 *February* 1948)

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

Table 1. *Amino-acids of media*

(g./l.)

	Amino-acid mixtures									
	X	*Y*	*B*	*C*	*D*	*I*	*N*	*O*	*P*	*R*
Glycine	—	—	1·4	1·4	1·4	2·0	1·4	2·0	2·0	2·0
DL-Alanine	—	—	0·5	0·6	0·6	0·2	0·6	0·6	—	—
DL-Valine	0·4	0·8	0·2	0·8	0·8	0·8	0·8	0·8	1·0	—
L-Leucine	—	0·8	0·2	0·8	0·8	0·8	0·8	1·0	1·0	1·0
DL-Isoleucine	0·8	0·8	0·4	0·8	0·8	0·8	0·4	0·6	1·0	1·0
DL-Proline	—	—	0·6	0·6	0·6	0·2	—	—	—	—
L-Hydroxyproline	—	—	0·2	0·2	0·2	0·2	—	—	—	—
DL-Phenylalanine	—	0·6	0·2	0·6	0·6	0·6	0·6	0·6	0·6	—
DL-Glutamic acid	—	—	0·3	0·4	—	—	—	—	—	—
DL-Aspartic acid	—	—	0·2	0·4	—	—	—	—	—	—
DL-Serine	—	—	0·2	0·2	0·2	0·2	—	—	—	—
L-Tyrosine	0·4	0·8	0·2	0·8	0·8	0·4	0·8	0·8	0·8	0·8
L-Histidine HCl (hydrate)	—	0·4	0·2	0·4	0·4	0·8	0·4	0·4	0·8	0·8
L-Arginine HCl	—	—	0·5	0·6	0·6	0·8	0·6	0·6	0·8	1·0
L-Lysine HCl	—	0·4	0·4	0·4	0·4	0·8	0·4	0·8	0·8	0·8
L-Tryptophan	0·4	0·4	0·2	0·4	0·4	0·8	0·4	0·6	0·8	0·8
DL-Threonine	0·4	0·6	0·2	0·6	0·6	0·8	0·4	0·6	1·0	1·0
DL-Methionine	—	0·6	0·4	0·6	0·6*	0·8	0·6	0·6	0·6	1·0
L-Cystine	0·4	0·02	0·2	0·02	0·02	0·02	0·02	0·06	0·04	0·02
Total concentration of amino-acids (%)	0·28	0·62	0·67	1·06	0·98	1·10	0·82	1·01	1·12	1·02

* Later increased to 1·0 g./l.

solution might result in extraneous growth-stimulating effects on the larvae.

The compositions of amino-acid mixtures are shown in Table 1.

Materials. Sodium caseinate was prepared from S.M.A. Co. 'Vitamin test' casein. Gelatin was Eastman Kodak purified calf-skin gelatin. Other materials used were prepared and purified by standard procedures.

L-3:5-Diiodotyrosine was provided by Prof. C. R. Harington and pteroylglutamic acid by Dr E. L. R. Stokstad, Lederle Laboratories.

RESULTS

Protein requirements of the mosquito larva

In media lacking protein or amino-acids, but complete in other respects, the mosquito larva is incapable of reaching even the second instar. Accordingly, its growth response to added protein or amino-acid mixtures is a sensitive index of the extent to which its exacting nutritional needs have been met in any particular medium. The provision of proteins in the solid state is complicated by the fact that often they cannot be maintained in finely divided form. In consequence the study of protein requirements was carried out with proteins in solution.

In order to determine the optimum concentration of protein for the mosquito larva, soluble casein was tested at various levels, with best results between 0·5 and 1%. Within this range the omission of glucose from the medium had, if anything, a beneficial effect. Lower concentrations of casein were inadequate to sustain growth (Table 2). The turbidity of soluble casein solutions after autoclaving, increasing as it did during the course of the experiment, made observation of the larvae difficult. We had recourse to sodium caseinate, which gave sparklingly clear media and at 1% concentration produced satisfactory growth and survival rates (Table 3).

Using these results as a standard of comparison, a number of other proteins and related substances were tested and it was clear that proteins known to

Table 2. *The optimum level of protein in the larval medium*

(Medium: *CM*, with changes as indicated.)

Concentration of soluble casein (%)	Concentration of glucose (%)	No. of second larval instars	No. of adult mosquitoes	Total time taken (days)	
				Mean	S.D.
0	2	0	—	—	
0·1	2	0	—	—	
0·2	0	19	0	—	
0·2	2	21	2	11·0	
0·5	0	17	11	11·5	±1·14
0·5	2	11	7	13·4	±1·21
1·0	2	33	20	11·0	±1·40
2·5	2	37	14	10·8	±1·32

Table 3. *The growth-promoting powers of various proteins*

(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.)

Protein tested (in solution in growth medium *CM*)	Concentration of protein (%)	Larval instars II No.	II Mean; S.D.	III No.	III Mean; S.D.	IV No.	IV Mean; S.D.	Pupae No.	Pupae Mean; S.D.	Adults Total time taken No.	Range	Mean; S.D.
Sodium caseinate	1·0	40	2·1±0·67	40	1·5±0·42	39	1·9±0·51	34	3·4±0·65	32	9·5–13·0	10·8±1·09
Ovalbumin	0·5	31	2·8±0·67	31	2·3±0·87	31	2·0±0·56	24	5·9±1·93	21	11·0–18·0	14·6±2·11
Gelatin	0·5	10	15·5±7·71	8	10·2±6·83	4	6·0	3	8·7	2	20·0–29·0	24·5
Gelatin	1·0	19	8·4±5·96	17	7·4±2·65	10	6·0±1·20	3	7·8	3	23·5–28·5	26·2
Edestin	1·0	20	2·6±0·69	20	2·1±0·45	20	2·4±1·09	18	5·4±1·71	17	10·5–20·5	14·3±1·93
Ovomucoid	1·0	21	4·6±1·43	20	5·3±2·52	18	6·9±3·50	10	8·8±2·19	7	18·0–33·0	22·8±4·64
Yeast nucleoprotein	1·0	14	3·3±1·48	12	1·5±0·32	12	1·9±0·30	10	4·1±0·62	10	11·0–16·5	13·1±1·72
Sericin (soluble)	0·5	0	No growth	—	—	—	—	—	—	—	—	—
Beef peptone	1–2	0	No growth	—	—	—	—	—	—	—	—	—
Tryptic digest of casein	1·0	22	4·1±1·70	22	2·8±0·78	22	2·8±0·69	20	4·1±0·77	20	13·5–18·5	15·6±1·41

be of inferior nutritive value for animals were equally unsatisfactory for the mosquito larva. Such nutritive inadequacy was revealed by decreased rates of growth and of survival (Table 3).

Casein digests and hydrolysates. With the exception of tryptic digest of casein which, as can be seen from Table 3, produced a good response, other casein digests and hydrolysates proved disappointing. Papain casein digest gave some stimulus to growth but acid hydrolysates were toxic, even after removal of chlorides and supplementation with amino-acids. In spite of all efforts to free the hydrolysates from humin and other possible toxic ingredients, they produced no growth.

Amino-acid mixtures. Preliminary studies were carried out with amino-acid mixtures used to supplement ovalbumin and gelatin. Details of the composition of these mixtures have been given in Table 1. Eleven amino-acids, in conjunction with 1% gelatin solution, produced a striking growth response (Table 4). With media containing amino-acids but no protein, one of the mixtures tested was *B*, consisting of 19 amino-acids in a concentration of 0·67%. Here growth was greatly delayed and survival poor. When the proportions were changed and the total concentration raised to 1·06% (mixture *C*) the results were more encouraging. Because of their possible toxic effects, aspartic and glutamic acids were next omitted and the amino-acid mixture *D* provided for excellent growth and survival. Mixture *I*, consisting of the same amino-acids in different proportions (more emphasis on essential amino-acids) was far less effective.

The next step was to omit amino-acids which, by analogy with requirements of higher organisms, might be expected to prove dispensable. The series of mixtures *N*, *O*, *P* and *R*, containing 14, 14, 13 and 11 amino-acids respectively in concentrations of 0·8–1·1% all gave results decidedly inferior to those obtained with mixture *D*. Mixtures *C* and *D* were also tested at one half of their previous concentrations. Although both media still contained

Table 4. *Growth of mosquito larvae on media containing mixtures of amino-acids*

Protein	Amino-acid mixture	No. of amino-acids in mixture	No. of second larval instars	No. of adult mosquitoes	Total time taken (days) Range	Mean S.D.
Ovalbumin (0·5%)	*X*	6	20	5	12·5–28	19·1
Gelatin (1·0%)	*X*	6	19	12	16·5–23	18·5±1·52
Gelatin (1·0%)	*Y*	11	21	21	10·5–15·5	12·5±1·05
Gelatin (1·0%)	*Y* without tyrosine	10	23	19	10·5–15·5	12·3±1·31
	B	19	21	10	18 –33	22·6±4·20
	C	19	20	14	10·5–16·5	13·4±1·65
	D	17	19	17	9·5–16	12·6±2·20
	I	17	22	13	16·5–26	22·7±2·83
	N	14	21	16	12 –26	15·5±3·15
	O	14	34	16	17 –29	23·3±3·67
	P	13	24	20	17 –23·5	19·3±2·56
	R	11	24	13	8·5–39·5	23·0±6·91
	C (half-strength)	19	23	13	16 –29·5	19·1±4·18
	D (half-strength)	17	18	14	14 –28	21·3±4·10

approximately 0·5% of amino-acids, growth and survival were adversely affected.

Attempts to eliminate yeast autolysate from media. In order to study the amino-acid requirements of the larva it was desirable that the basal medium should be free from such amino-acids as might be introduced by the yeast autolysate. Efforts were made to replace the autolysate by pteroylglutamic acid and the lactone of 4-pyridoxic acid which, as reported by us (Golberg & De Meillon, 1947) are capable of producing good growth in sodium caseinate media. Replacement of sodium caseinate by amino-acid mixtures such as *D* failed to produce growth, although there was no evidence of toxicity.

Attempts to concentrate the active principles from yeast autolysate by methods previously used for urine (Golberg, De Meillon & Murray, 1947) were successful only as long as caseinate was used in the larval medium, and only in the initial stages of the concentration process.

One further possibility was a reduction in the concentration of yeast autolysate, from what might appear to be the unduly high level of 2%. Since each experiment often lasted for well over a month, the deterioration of the medium in the course of prolonged incubation made it necessary to have an initially high level of the unstable growth factors.

Amino-acid requirements

With amino-acid mixture *D* as the starting point, each component amino-acid was omitted in turn. For convenience the results have been recorded separately in Tables 5 and 6, according as the amino-acids omitted proved to be 'dispensable' or 'indispensable'. The larval response was by no means as clear-cut as this division would suggest. In every case the survival rate was smaller and the rate of growth to a greater or lesser extent delayed. In some cases the tests were also carried out using as the basal medium mixture *I*, which contained much smaller proportions of 'dispensable' amino-acids. This time the omission of individual 'dispensable' amino-acids made little difference to the larval response.

In contrast to the somewhat ambiguous results with 'dispensable' amino-acids, it was quite clear that the following were quite indispensable: glycine, L-leucine, DL-isoleucine, L-histidine, L-arginine, L-

Table 5. *The effects on growth of mosquito larvae of the omission of a single amino-acid*

Amino-acid mixture	Amino-acid omitted	No. of second larval instars	No. of adult mosquitoes	Total time taken (days)	
				Range	Mean S.D.
D	DL-Alanine	23	13	15·5–27·5	19·5±3·61
I	DL-Alanine	19	9	19·5–31·5	25·6±3·42
D	DL-Valine	21	9	13·5–25	18·7±3·0
I	DL-Valine	21	15	16 –25	19·6±2·34
D	DL-Proline	17	12	14 –21	16·5±1·89
I	DL-Proline	18	10	18 –28·5	24·5±2·07
D	L-Hydroxyproline	17	7	11 –22	13·9±3·52
I	L-Hydroxyproline	22	14	19 –30·5	25·7±3·13
D	DL-Serine	21	9	15 –18	15·7±1·25
I	DL-Serine	20	12	18·5–31	26·0±3·39
D	L-Cystine	34	15	14 –27·5	19·7±3·03
D	L-Tyrosine	20	11	16·5–24·5	20·5±2·74
D	DL-Phenylalanine	20	10	11 –19·5	13·5±2·52
N	DL-Phenylalanine	20	17	12·5–28·5	19·8±4·06

Table 6. *The effects on growth of mosquito larvae of the omission of amino-acids**

Amino-acid mixture used	Amino-acids omitted	Larval instars II No.	II Mean; S.D.	III No.	III Mean; S.D.	IV No.	IV Mean; S.D.	Pupae No.	Pupae Mean
O	Glycine	2	5·0	0	—	—	—	—	—
D	Glycine	22	7·3±1·67	22	10·5±2·12	6	7·6±1·94	0	—
O	L-Leucine	16	9·1±2·74	3	10·3	0	—	—	—
D	DL-Isoleucine	19	4·4±1·17	18	4·0±0·91	5	7·1±1·02	0	—
O	L-Histidine	0	—	—	—	—	—	—	—
O	L-Arginine	0	—	—	—	—	—	—	—
O	L-Lysine	18	9·8±3·07	12	8·7±1·89	0	—	—	—
D	L-Tryptophan	14	7·0±2·77	14	8·0±3·41	4	7·5±1·66	1	11·0
O	DL-Threonine	17	8·6±2·91	5	6·1±2·20	0	—	—	—
D	DL-Phenylalanine and L-tyrosine	0	—	—	—	—	—	—	—
D	DL-Methionine	3	11·3	0	—	—	—	—	—

* For explanation of headings see Table 3.

lysine, L-tryptophan, DL-threonine, DL-methionine. The case of DL-phenylalanine and L-tyrosine will be considered below.

The sulphur-containing amino-acids. In the experiments just described a curious phenomenon was observed in media from which cystine was omitted. Despite the presence of large amounts of DL-methionine in each medium, the great majority of the adults formed from the pupae were found dead in the medium, with only the upper half of the body projecting from the pupal case. Examination of the adults failed to reveal any obvious malformations. In addition, the growth and survival rates were reduced and could not be improved by the addition of extra amounts of methionine or DL-homocystine or glutathione. However, in the case of glutathione all the adults emerged, apparently quite normally (Table 7).

Glycine and arginine. Attempts to replace glycine by other compounds were partially successful in the case of glutathione but not when DL-serine or creatine were used. Similarly, L-arginine could be replaced to some extent by DL-citrulline, to a slight extent by L-ornithine, but not at all by creatine (Table 8).

Phenylalanine and tyrosine. In the experiments recorded in Table 5, neither L-tyrosine nor DL-phenylalanine alone could be shown to be essential, although the omission of both prevented all growth (Table 6).

Effects on cuticular pigmentation

The mosquito larva possesses chitinous plates on the head, siphon and saddle, which become more extensive as the larva approaches pupation. It is these circumscribed areas which are darkly pigmented from the second to the fourth instar, in contrast to the more diffuse pigmentation of the pupa. In most media the shade of pigmentation of the larvae varied from deep brown to jet black, but on one occasion it was noted that larvae grown on a papain liver digest medium were extraordinarily pale (Golberg, De Meillon & Lavoipierre, 1945). At the time this observation could not be repeated.

In the present series of experiments pale, unpigmented larvae were first observed in media con-

Table 7. *Effects of sulphur-containing amino-acids on the growth of mosquito larvae*

(Amino-acid mixture D used in basal medium.)

Additions to medium	No. of second larval instars	No. of adult mosquitoes	Total time taken (days) Range	Mean	S.D.
(a) Methionine omitted from amino-acid mixture *D*					
None	3	0	—	—	
L-Cystine (0·4 g./l.)	13	2	17–22	19·5	
L-Cystine (0·4 g./l.) + choline (0·8 g./l.)	14	1	—	19	
DL-Homocystine (0·6 g./l.) + choline (1 g./l.)	20	2	—	24	
DL-Homocystine (1·0 g./l.) + choline (1 g./l.)	8	0	—	—	
DL-Homocystine (1·0 g./l.) + choline (1 g./l.)	0	—	—	—	
(b) Cystine omitted from amino-acid mixture *D*					
None	34	15*	14 –27·5	19·7	±3·03
DL-Methionine (0·4 g./l.)	15	4†	17·5–24	20·0	±2·47
DL-Homocystine (0·06 g./l.)	21	12‡	15·5–33·5	21·5	±5·11
Glutathione (0·2 g./l.)	18	9	17 –25·5	21·2	±3·15

Numbers of adult mosquitoes which died while emerging: * = 11, † = 4, ‡ = 10.

Table 8. *Effect of arginine and glycine on the growth of mosquito larvae*

Compound added	No. of second larval instars	No. of adult mosquitoes	Total time taken (days) Range	Mean	S.D.
(a) L-Arginine omitted from amino-acid mixture *O*					
—	0	—	—	—	
Citrulline (0·6 g./l.)	21	11	16–30·5	21·4	±4·57
Ornithine (0·6 g./l.)	22	1	—	29·5	
Creatine (0·8 g./l.)	0	—	—	—	
(b) Glycine omitted from amino-acid mixture *O*					
—	2	0	—	—	
Serine (0·2 g./l.)	11	0	—	—	
Creatine (1·0 g./l.)	4	0	—	—	
Glutathione (1·4 g./l.)	17	5	17–26	23·7	±3·52

taining gelatin as the sole source of protein. The ghost-like appearance of the larvae was due to lack of pigment in the chitinous areas described. Supplementation with amino-acids, including phenylalanine and/or tyrosine restored the pigment. An interesting feature of these experiments was the fact that the pale larvae gave rise to pupae which were partially pigmented and, as far as could be ascertained, the adults possessed their full characteristic pigmentation. It was also noted that lack of pigmentation had little, if any, definitely adverse effect on growth or survival (Table 5).

In the case of amino-acid mixture *D* the omission of tyrosine produced pale larvae and almost completely unpigmented pupae, but the omission of phenylalanine appeared not to affect pigmentation. This apparent paradox was resolved by increasing the level of phenylalanine in the tyrosine-free medium. At a level of 1·0 g. DL-phenylalanine/l. of medium the larvae were pale up to the third instar, but the fourth-instar larvae were dark and the pupae appeared normal. At a level of 1·4 g. of DL-phenylalanine pigmentation was normal throughout.

Intermediate degrees of pigmentation. Observation of fourth-stage larval pelts or, where these were not available, of early instar larvae, indicated the existence of the following intermediate degrees of pigmentation between the colourless larva and the dark normal shade:

0 White, with no pigment visible.
1 Siphon and saddle clear yellow; no darkening.
2 Siphon and saddle bright yellow; no darkening.
3 Slight darkening of siphon and saddle.
4 Siphon definitely darkened; saddle with a large, clearly marked area dorsally.

It was thus a matter of interest to us to assess the shades of pigmentation resulting from the use of various possible precursors of melanin in a basal medium capable of producing colourless larvae but fairly good growth and survival. Some of the compounds tested were too toxic to permit growth to the fourth instar, but the remainder gave rise to varying degrees of pigmentation (Table 9).

DISCUSSION

Insects offer a striking contrast to higher animals in that many can dispense with dietary nitrogen during adult life (Uvarov, 1928). The proteins utilized during this time must be drawn from reserves accumulated during larval life. An additional factor to be taken into consideration in many instances is the rapid rate of growth. These factors operating together ensure that the mosquito larva is highly exacting with regard to its protein requirements. Failure to obtain protein prevents growth even to the second instar. Failure to obtain protein of adequate quality may have the same effect or may prolong the time of development far beyond its normal span.

For optimum growth in our media the mosquito larva requires a level of protein as high as 1 % and, apparently, equally high concentrations of total amino-acids. In earlier experiments (Golberg *et al.* 1945) we had found that a very light autoclaved suspension of micro-organisms sufficed for excellent growth of the larvae. In fact, it was frequently observed in contaminated media that before the presence of the contaminants was visible to the naked eye it was betrayed by a distinct acceleration of larval growth. The high level of protein and of amino-acids found necessary in our experiments may be a consequence of the nature of the media employed or of the fact that the mosquito larva is equipped physiologically to deal with discrete particles rather than solutions.

In this connexion attention should be drawn to the remarkably high levels of amino-acid nitrogen observed in the blood of insect larvae and chrysalids (Bishop, Briggs & Ronzoni, 1925; Courtois, 1928; Duval, Portier & Courtois, 1928). Values as high as 4 g. amino-nitrogen/l. have been reported, corresponding to well over 20 g. of amino-acids/l. of haemolymph. Such a consequence of histolytic changes probably obtains in the mosquito larva and would explain the necessity for the high level of dissolved protein or amino-acids.

Table 9. *Pigmentation induced in mosquito larvae by various compounds*

(Medium contained 0·5 % gelatin and amino-acid mixture *Y* with tyrosine and phenylalanine omitted. All compounds tested at a concentration of 0·4 g./l.)

Compound tested	Specimens examined	Degree of pigmentation*
DL-Phenylalanine	Fourth-stage pelts	1
L-Tyrosine	Fourth-stage pelts	4
Tyramine hydrochloride	Fourth-stage pelts	1
L-3-Aminotyrosine	Fourth-stage pelts	2
L-3:4-Dihydroxyphenylalanine	Third-stage larvae	0
DL-α-Aminophenylacetic acid	Fourth-stage pelts	1
p-Aminophenylacetic acid	Fourth-stage pelts	2–3
L-3:5-Diiodotyrosine	Third-stage larvae	0
Adrenaline	Second-stage larvae	0

* The significance of the numbers used is explained in the text, p. 384.

The mosquito larva is exacting in its requirement for 'complete' proteins. In this respect it resembles *Drosophila* (Lafon, 1938), *Blatella* (McCay, 1933) and *Pyrausta* (Bottger, 1942). Lafon & Teissier (1939) have drawn attention to the suitability of yeast proteins for the nutrition of *Tenebrio*, and their remarks apply to many other insects, including the mosquito larva.

Our inability to use casein hydrolysates for the mosquito larva recalls a similar experience reported by van't Hoog (1935) with *Drosophila*. Lafon (1938) and Tatum (1941) met the protein needs of *Drosophila* by means of casein hydrolysates supplemented by cystine and/or tryptophan. On the other hand, Kozhanchikov (1944), Lafon (1938) and Buddington (1941) met with little or no success in their efforts to replace proteins by amino-acids in insect diets. Our results with mixtures of amino-acids show clearly that it is not only the qualitative but also the quantitative composition of an amino-acid mixture which determines its growth-stimulating ability. By demonstrating the improvement in the rates of growth and survival which is brought about by the presence of non-essential amino-acids in adequate proportions these experiments lay emphasis on the antagonistic and toxic effects of amino-acids (Gladstone, 1939; Hutchings & Peterson, 1943; Pelczar & Porter, 1943; Porter & Meyers, 1945).

The suggestion that strepogenin (Woolley, 1946 and earlier references) is required by the mosquito larva would serve to explain the fact that growth on amino-acid mixtures is in general inferior to that which results from the use of casein; and that pteroylglutamic acid and the lactone of 4-pyridoxic acid can produce growth with sodium caseinate but not with amino-acids. However, proteins such as egg albumin and gelatin, known to contain little strepogenin (Sprince & Woolley, 1945), produced slow growth; and when supplemented with amino-acid mixture *Y*, gelatin led to growth not greatly inferior to that obtained with casein.

Essential amino-acids. In connexion with larval requirements of amino-acids the concept of 'essentiality' has a special significance, since provision must be made not only for growth but at the same time for maintenance of the adult. It is interesting to observe, however, how closely the requirements of the larva resemble those of higher animals, in particular those of the chick. Thus glycine and arginine are indispensable amino-acids for the chick (Klose, Stokstad & Almquist, 1938; Almquist & Mecchi, 1940; Hegsted, Hier, Elvehjem & Hart, 1941); they are also indispensable for the mosquito larva. The chick can utilize citrulline but not ornithine in place of arginine (Klose & Almquist, 1940; Klose *et al.* 1938). The mosquito larva is able to grow and metamorphose when given citrulline; it does utilize ornithine to a small extent, since there was delayed growth to the fourth instar. (The maximum times recorded were: to the second stage, 18·5 days; to the third, 20 days; to the fourth, 15 days; to the pupa, 12 days. Many larvae were still alive when the experiment was discontinued.) There is a striking similarity between these observations and the growth of *Streptococcus haemolyticus* on a medium in which arginine was replaced by ornithine: Gale (1945) reported that with ornithine growth was only one quarter of that with arginine and was also irregular. Although isotope experiments have demonstrated the conversion of L-serine into glycine in the rat and guinea pig (Shemin, 1946), Almquist & Grau (1944) found that the omission or inclusion of serine in chick diets had no effect on their need for glycine. Similarly, the mosquito larva was unable to utilize serine in place of glycine. With glutathione its response was definite but delayed and incomplete. In contrast to its effectiveness in the chick (Almquist, Mecchi & Kratzer, 1941; Hegsted *et al.* 1941) creatine proved incapable of replacing glycine in the larval diet.

Little is known concerning the role of glycine in invertebrates. Kutscher & Ackermann (1933) noted the surprising fact that glycine betaine has not been found in insects, although it definitely occurs in Crustacea. They attributed this observation to the rapidity of insect metabolism, which prevents the accumulation of glycine in appreciable quantity and hence the formation of betaine.

Valine. Microbiological assay of valine using *Lactobacillus arabinosus* revealed a concentration of 29 mg. valine per g. of yeast autolysate. It is doubtful whether in such a case the entire response of the test organism was due to valine, but the result accounts for the finding that growth of the mosquito larva is not seriously affected by lack of added valine in the medium. A final decision on the essentiality of valine for the mosquito larva will have to be deferred until the existing difficulties in devising a suitable test medium have been overcome.

Sulphur-containing amino-acids. That cystine is a dispensable amino-acid is generally accepted. So much so, that most investigators of recent years have made no effort to ascertain whether in fact cystine is replaceable in all its functions by methionine. Hegsted (1944) acknowledged this omission in studies on the amino-acid requirements of chicks. There exists considerable evidence, especially among micro-organisms, that cystine plays an indispensable part in nutrition in certain circumstances. Such observations give added point to the effect of cystine lack on the mosquito larva. The failure of a large proportion of adults to emerge successfully suggests that a deficiency of cystine is possible, despite the high level of methionine in the medium. Moreover, while cystine and homocystine are both capable of replacing methionine to some extent, only gluta-

thione could replace cystine in its ability to promote normal emergence of adults.

Phenylalanine and tyrosine. Mosquito larvae seem equally capable of utilizing either phenylalanine or tyrosine, a fact which might be explained by the presence of a small quantity of phenylalanine in the yeast autolysate used. This suggestion fails to account for the fact that growth is delayed in the absence of tyrosine but remains unaffected when phenylalanine is omitted from the medium.

The production of unpigmented larvae on media lacking tyrosine or phenylalanine is, to our knowledge, the first demonstration that the absence of these amino-acids from the diet can affect pigmentation. It is instructive to consider the quantitative aspect of these experiments. Since all media contained equal amounts of yeast autolysate, this factor remained constant. From the values given by Hodson & Krueger (1946), a 1 % solution of sodium caseinate would contain 0·48 g. L-phenylalanine and 0·53 g. L-tyrosine/l. This medium produces full pigmentation. In an amino-acid medium, DL-phenylalanine, in a concentration of 1·4 g./l., also produces darkly pigmented larvae; but a level of 1·0 g./l. allows pigmentation of the fourth-stage larva but not of the earlier instars; 0·6 g./l. produced no visible pigmentation. In 0·5 % gelatin the amount of phenylalanine present would be 0·13 g./l. (calculated from the analysis of Block, Jervis, Bolling & Webb, 1940). An additional 0·4 g./l. of L-tyrosine is sufficient for the dark pigmentation of fourth instar larvae; but the same amount of DL-phenylalanine produces pale yellow pigmentation. Thus both with amino-acid mixture *D* and with gelatin it is clear that L-tyrosine is greatly superior to DL-phenylalanine in its ability to promote the formation of melanin. It may be that D-phenylalanine is not utilizable for melanin formation—*in vivo* only L-3:4-dihydroxyphenylalanine is acted upon by dopa oxidase (Sumner & Somers, 1943). Taking into account the strong melanin-forming powers of *p*-aminophenylacetic acid (Table 9) it seems more probable that DL-phenylalanine is utilized for the most part for other, more essential, purposes.

The activity of *p*-aminophenylacetic acid, and even to a small extent of α-aminophenylacetic acid, indicates that compounds of various types must be taken into consideration as precursors of melanin *in vivo*. The findings of Pryor, Russell & Todd (1946) that the phenolic substance responsible for the hardening of the cockroach ootheca is protocatechuic acid, in addition to the results of earlier workers, suggests that a wide range of compounds may possibly play a part in the darkening of the insect cuticle.

Serra (1946) has shown that melanins extracted from hairs of different hues contain varying proportions of melanoid pigment and protein. According to his analyses, a combination of 60 % melanoid pigment and 40 % protein leads to a black melanoprotein, but 30 % pigment and 70 % protein constitutes a yellow melanoprotein. The colours observed in the head, siphon and saddle of the mosquito larva are therefore indicative of the relative proportions of melanoid pigment formed under the experimental conditions employed. From our results it becomes clear that the production of melanin is a process depending directly on the level of dietary phenylalanine and tyrosine—or, more correctly, on the excess over the quantities required for growth and protein synthesis. In consequence of this fact, varying degrees of melanic pigmentation can be produced at will and absence of pigmentation during the early instars, when all available phenylalanine is presumably required for protein synthesis, can be followed by the production of some melanin when the larva is full-grown.

Our observation that in the mosquito larva pigmentation has little connexion with growth underlines the statement made by Wigglesworth (1942) that 'the pigmented constituents of insects, in some cases...are, perhaps, substances of physiological importance; but the majority seem to be merely by-products of metabolism'. Since tanning by means of a melanin precursor plays an essential part in the hardening of the insect cuticle (Pryor, 1940; Trim, 1941; Hurst, 1940, 1945) it is possible that in unpigmented larvae the cuticle remains soft. The physiological implications cannot be discussed here. There is obviously a wide scope for the use of the nutritional approach to the study of melanin formation *in vivo* for the elucidation of the many problems as yet unsolved in this field.

SUMMARY

1. In a medium complete in other respects, but lacking protein the mosquito larva does not grow to the second instar.

2. After yeast, casein is the most suitable protein, but incomplete proteins, on supplementation with amino-acids, prove adequate for growth and survival to the adult stage.

3. Good rates of growth and survival could be produced by using mixtures of amino-acids, but variation in their relative proportions or reduction in the number of amino-acids had adverse effects.

4. By omitting single amino-acids from a mixture, it was possible to establish that the following are essential for the mosquito larva: glycine, L-leucine, DL-isoleucine, L-histidine, L-arginine, L-lysine, L-tryptophan, DL-threonine, DL-phenylalanine and DL-methionine. The status of DL-valine could not be determined while using yeast autolysate in the medium.

5. There was evidence that the omission of L-cystine resulted in a high proportion of adult mosquitoes failing to emerge.

6. According to the level of phenylalanine or tyrosine in the medium various shades of pigmentation could be produced in the mosquito larvae. The adults emerging even from wholly unpigmented larvae were normally pigmented and there appeared to be little relation between pigmentation and growth or survival. A number of compounds were found capable of producing intermediate degrees of pigmentation in the mosquito larva.

We wish to thank Prof. C. R. Harington and Dr E. L. R. Stokstad for materials used in these investigations. It is a pleasure to acknowledge the assistance of Miss M. Currie and Mr J. M. Thorp.

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The Chemistry of Connective Tissues

1. THE STATE OF COMBINATION OF CHONDROITIN SULPHATE IN CARTILAGE

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(*Received* 17 *February* 1948)

In recent years evidence has been presented by several groups of workers that chondroitin sulphate exists in tissues such as cartilage in a very highly polymerized condition. The early workers in the field regarded chondroitin sulphate as an oligosaccharide of quite low molecular weight, and Levene (1925) formulated the substance as a non-reducing tetrasaccharide composed of two residues of glucuronic acid and two residues of a sulphate of *N*-acetylglucosamine. Perhaps the main reason for the failure of the early workers to recognize the highly polymerized character of the polysaccharide lay in the fact that strongly alkaline reagents were invariably used to effect the initial extraction from the tissue, and it has since been shown that the polysaccharide is rapidly degraded in the presence of strong alkali, particularly at temperatures above 0°.