CHANGES IN THE FREE AMINO ACIDS OF THE BLOOD OF BLOWFLY LARVAE AT METAMORPHOSIS

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Summary

Blood from the larval, prepupal, and early pupal stages of *Calliphora augur* (F.) contains the same 18 free amino acids. In addition, hydroxyproline is present in larval and prepupal blood. The quantitative differences in the concentrations of these amino acids are reported. Larval blood has the highest free amino acid content (6·6 mg/ml) followed by early pupal blood (4·6 mg/ml) and prepupal blood (3·3 mg/ml). As the larva matures to the prepupa, the greatest decreases occur in the concentrations in the blood of alanine, glutamic acid, glutamine, glycine, proline, and tyrosine. The metabolic significance of these changes are discussed.

I. INTRODUCTION

The free amino acid content of the blood of insects is very high in comparison with that of other animals, and from 5 to 20 mg/ml are commonly found. Until the advent of paper chromatography few of the amino acids had been identified, but since about 1949 knowledge of the amino acids in the blood has begun to accumulate more rapidly. Even so, very little has been published on the qualitative and quantitative differences in the amino acid composition of the blood of insects at different times during their life-cycle. Reference can be made to the work of Auclair (1953) on *Galleria mellonella*, Sarlet, Duchateau, and Florkin (1952) on *Bombyx mori* (L.), and Drilhon (1952) on *Macrothylacea rubi* L., although there are other papers in which changes in the composition of only one or two amino acids are given.

In the absence of confining blood vessels the blood of insects is free to flow among the organs and tissues within the body cavity. The blood is the reservoir for the products required for and produced by nearly every physiological activity of the insect body, and in growing or metamorphosing insects the changes in blood composition reflect the morphogenetic and biochemical transformations taking place in the tissues. Just prior to pupation, the larval cuticle of blowflies increases considerably in thickness. In the early pupal stage this cuticle is hardened and becomes dark in colour. Changes in the free amino acids and phenols of the blood at metamorphosis may therefore lead to an understanding of the nature of these processes.

II. EXPERIMENTAL

(*a*) *Preparation of Samples*

A culture of *Calliphora augur* (F.) was established from adults collected in the field. The larvae were reared on sheep's liver in a room maintained at 25°C. Twenty-four hours prior to the collection of larvae for experimental purposes the adult flies were deprived of liver. Liver was then placed in the cages and the larvae (approx.

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2000) laid over a period of 30 min were collected. These larvae were provided at all times with an abundant supply of liver. After 72 hr the larvae were almost fully grown but were still actively feeding. By the time the larvae were 96 hr old they had left their food, had voided their gut contents, and entered what may be called the prepupal stage. 2–3 days later the larva becomes immobile and shortly afterwards hardens and darkens to form the puparium. Blood samples were taken from the almost fully grown feeding larvae (72 hr old) (in future called larval blood), from the prepupae (96 hr old), and from early pupae. For the latter, immobile individuals were selected in which the puparium was not coloured or had only just begun to colour.

Before blood samples were collected, the various stages were washed in several changes of distilled water and dried on filter paper. Blood was collected by piercing the anterior end of the animal with a needle and collecting, on the end of a small spatula, the drop of blood which appeared. The blood was placed immediately under light petroleum (boiling range 40–60°C) to prevent it darkening. Approximately 2 ml of blood was collected from 200 larvae or 200 prepupae but considerably less blood was obtained from the early pupal stage. A convenient tool to use when collecting the blood samples was a length of stainless steel wire ($\frac{1}{16}$ in. dia.) sharpened to a needle point at one end and flattened to form a small spatula at the other end. Although by this method it was not possible to collect all the blood from each animal, one could be sure that the blood which was collected was uncontaminated by other tissues.

Each of the samples of blood was treated as follows: the blood, under light petroleum, was centrifuged, and a known volume removed and mixed with an equal volume of 10 per cent. trichloroacetic acid to precipitate the proteins. After 1 hr the suspension was centrifuged and the supernatant poured into a stoppered tube. The precipitate was washed with three lots of 2 ml of 5 per cent. trichloroacetic acid when the extract no longer gave a positive ninhydrin test for amino acids. The combined washings and original supernatant were freed from trichloroacetic acid by repeated extraction with ether. The aqueous solution was evaporated to dryness *in vacuo* over phosphorus pentoxide at room temperature and the residue dissolved in phosphate buffer of pH 7 (0·04M $Na_2HPO_4.2H_2O$ and 0·027M KH_2PO_4) containing 10 per cent. *iso*propanol. The volume of buffer used was equal to the original volume of blood taken. Hydrogen peroxide (100 vol., one-fifth of the volume of the sample taken) was added to a sample of each protein-free specimen of "blood", the solution allowed to stand for several hours, evaporated to dryness *in vacuo*, and the residue taken up in a volume of buffer equal to that of the sample taken. This treatment converted any cystine or cysteine to cysteic acid and any methionine to methionine sulphone.

(*b*) *Paper Chromatography*

(i) *Qualitative Analysis of Amino Acids.*—The six solutions representing the three samples of blood were subjected to qualitative paper chromatography using both the one- and two-dimensional methods. The following solvents were used for development of the chromatograms: 75 per cent. aqueous phenol; phenol buffered to pH 10 (74 per cent. phenol, 26 per cent. buffer (0·053M boric acid and potassium chloride,

0·047M sodium hydroxide)); 60 per cent. aqueous acetone; acetone buffered to pH 7 (60 per cent. acetone and 40 per cent. buffer (0·04M $Na_2HPO_4.2H_2O$ and 0·027M KH_2PO_4)); 65 per cent. aqueous pyridine; and *n*-butanol–acetic acid–water (77:6:17 by volume). Where buffer solutions were used the paper was treated with the same buffer solution before use. The buffer solution was allowed to flow on to the paper from a pipette until the paper was saturated. The paper was air dried before use. For two-dimensional runs various combinations of these solvents were used in order to obtain satisfactory separation of all amino acids. In addition to ninhydrin the following special reagents were used to detect the spots corresponding to the amino acids: for proline and hydroxyproline—isatin (Hackman and Lazarus 1956); for histidine—diazotized sulphanilamide (Hackman and Lazarus 1956); and for taurine—*o*-phthaldehyde (Curzon and Giltrow 1954). Hydrolysis (6N HCl in a sealed tube at 110°C for 24 hr) of the deproteinized blood samples did not produce any new compounds.

(ii) *Quantitative Analysis of Amino Acids.*—The amino acids present in the six solutions representing the samples of blood were estimated by the quantitative paper chromatographic method of Hackman and Lazarus (1956) using five replicates. The samples which had been treated with hydrogen peroxide were used for the phenol solvent, the other samples for the remaining three solvents.

(iii) *Phenols.*—The samples of blood were examined for phenolic compounds. Benzene–acetic acid–water (40:40:20 by volume), chloroform–acetic acid–water (50:25:25 by volume), *n*-butanol–phenol–water (1:9:10 by weight), and an aqueous solution of carbon dioxide of pH 4·2 were used as solvent systems for development of the paper chromatograms. To detect spots due to phenols the following reagents were used: Folin-Denis reagent, Folin's reagent, Millon's reagent, and Pauly's reagent (Dalgliesh 1952) and ferric chloride and James' ferricyanide reagent (James 1948; James and Kilbey 1950). No phenolic compounds other than tyrosine were detected.

III. Results and Discussion

The results of the qualitative and quantitative analysis of the free amino acids present in the blood of *C. augur* at three different stages of its life-cycle are given in Table 1. By "trace" is meant an amount which was too low to be estimated conveniently even after concentrating the solutions or by multiple spotting (not greater than 0·1 mM). Tryptophan, histidine, β-alanine, ornithine, and β-(3,4-dihydroxyphenyl)alanine could not be detected in any of the blood samples. A single sample of blood from some 200 individuals of each stage was used for the analysis. However, many qualitative analyses (paper chromatography) have been performed on different samples of blood corresponding to each stage and in every case the same amino acids were detected and, as far as could be judged, the variations in amounts present were similar.

Blood from the larval, prepupal, and early pupal stages contains the same 18 free amino acids. In addition hydroxyproline is present in larval and prepupal blood. The free amino acid content of the larval blood amounts to about 6·6 mg/ml, of prepupal blood 3·3 mg/ml, and of early pupal blood 4·6 mg/ml. Considering larval and prepupal bloods only, the greatest decreases occur in the concentrations of alanine,

glutamic acid, glutamine, glycine, proline, and tyrosine. Although the concentration of aspartic acid in prepupal blood is only half that in larval blood the concentrations are very low. During the pupal stage, larval tissues are being broken down and the adult fly is being formed. Consequently it would be very difficult to correlate changes in amino acid content with any particular metabolic process.

Amino acids serve as units for the synthesis of proteins or they may be metabolized. They must make some contribution to the osmotic pressure of the blood and they must have some buffer action even if only a minor one. An early step in the breakdown of amino acids is the loss of the amino groups which may be stored,

TABLE 1

AMINO ACID COMPOSITION OF THE BLOOD OF CALLIPHORA AUGUR AT DIFFERENT STAGES DURING ITS LIFE-CYCLE

Amino Acid	Concentration (mM) ± Standard Error:		
	Larval Stage	Prepupal Stage	Early Pupal Stage
Alanine	13·29±0·49	4·88±0·13	3·44±0·12
Arginine	Trace	Trace	Trace
Asparagine	2·65±0·17	2·22±0·15	1·89±0·16
Aspartic acid	1·08±0·11	0·57±0·10	0·98±0·11
Cysteine and/or cystine	Trace	Trace	Trace
Glutamic acid	3·27±0·14	1·65±0·15	4·28±0·15
Glutamine	7·45±0·42	3·12±0·18	4·42±0·40
Glycine	3·93±0·10	1·82±0·11	3·25±0·10
Hydroxyproline	Trace	Trace	—
Leucine and/or isoleucine	1·51±0·06	1·46±0·06	0·84±0·06
Lysine	Trace	Trace	Trace
Methionine	Trace	Trace	Trace
Phenylalanine	1·18±0·12	1·16±0·12	1·76±0·11
Proline	8·01±0·20	1·80±0·04	5·50±0·11
Serine	2·43±0·08	1·61±0·09	2·93±0·09
Taurine	Trace	Trace	Trace
Threonine	Trace	Trace	Trace
Tyrosine	7·41±0·05	3·94±0·03	5·23±0·03
Valine	1·36±0·05	1·30±0·05	0·87±0·04

transferred to a suitable compound (transamination), or excreted as ammonia, urea, or uric acid. Amino groups are stored by reaction with glutamic acid and aspartic acid to form glutamine and asparagine respectively. The amino acids principally concerned in transamination reactions are alanine, aspartic acid, and glutamic acid when the amino group is transferred to the corresponding keto acid, pyruvic, oxalacetic, or α-ketoglutaric acids. In the light of present evidence the two α-aminodicarboxylic acids appear to be the most direct link between the general metabolism of the cell and the amino acids. Glycine is an extraordinarily active substance, participating in many activities of the cell over and above its role as a protein constituent. It is a unit of several fundamentally important cell constituents, and is

either an end-product or a precursor of substances which enter into diverse metabolic activities. The wide range of metabolic channels through which glycine is routed is given by Weinhouse (1955).

Reference to the work of Dennell (1946) on the larval cuticle of *Sarcophaga falculata* Pand. shows that in the period just prior to the prepupal stage (third to the fourth day) the cuticle undergoes a very considerable increase in thickness (from approx. 40–180μ). This increase in thickness is due to the formation of new cuticular substance and not to the absorption of water. The same is true of *C. augur* larvae. It is therefore to be expected that metabolism is at a high level throughout this period and that protein, chitin, polyphenols, and other cuticular components are being synthesized. Consequently there would be changes in the concentrations of alanine, glutamic acid, aspartic acid, glutamine, asparagine, and glycine in the two periods represented by the larval and prepupal bloods, although many other amino acids could be involved in metabolic processes. These amino acids could be incorporated directly in the cuticular proteins, and they could be taking part in other related metabolic processes. Proline could be incorporated directly in the cuticular protein, for it is known that insect cuticular proteins have a higher proline content (Hackman 1953*a*) while the overall conversion of proline into glutamic acid is well established.

Tyrosine may also be incorporated in the proteins of the cuticle for these proteins have a high tyrosine content (approx. 11·5 per cent. (Trim 1941; Hackman 1953*a*)). However, there is strong evidence to suggest that tyrosine in the blood is the precursor of the *o*-dihydric phenols which provide the quinones necessary for the tanning (i.e. hardening and darkening) of the larval cuticle to form the puparium (Dennell 1947; Fraenkel and Rudall 1947; Pryor, Russell, and Todd 1947; Hackman 1953*b*). A marked decrease in tyrosine content of the blood as the fully grown larva approaches pupation is to be expected. Dennell (1947) has brought forward evidence to show that in *S. falculata* the blood of the early pupa contains an *o*-dihydric phenol. However no phenolic compounds, other than tyrosine, could be detected in the larval, prepupal, or early pupal blood of *C. augur*.

Relatively little is known of the origin of D-glucosamine, *N*-acetyl-D-glucosamine, and chitin. From the observations of Leloir and Cardini (1953) and Lowther and Rogers (1955) it appears likely that glutamine is concerned in the biosynthesis of D-glucosamine and therefore of chitin which is built up of *N*-acetyl-D-glucosamine residues. Ussing (1946) has suggested that glutamine may function in the synthesis of uric acid in insects.

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