DEVELOPMENTAL CHANGES IN THE LATE LARVA OF *CALLIPHORA STYGIA*

I. HAEMOLYMPH

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Summary

Quantitative studies of plasma levels of protein, lipid, and soluble carbohydrate in larvae of *C. stygia* reveal discontinuities at days 6 and 10 of development. From day 3 to 6 plasma levels of protein and lipid increase sharply. After day 6, there is a decline in the concentration of plasma protein which is accelerated at the onset of pupation. The plasma lipid concentration rises abruptly at day 10, but then declines with the onset of puparium formation. The concentration of soluble carbohydrates in the haemolymph falls slightly between days 6 and 7 of development and commences to rise at about day 10; still higher levels are reached in the quiescent larva and early prepupa.

Determinations of haemolymph volume were made at intervals throughout third instar, using a dye-dilution method. The mean haemolymph volume per larva increases rapidly from 25 μl at day 4 to 53 μl at day 6, and thereafter falls gradually to 43 μl in the quiescent larva.

Acrylamide gel electrophoresis of plasma protein permits consistent resolution of at least 25 bands in third-instar larvae and 14 in 3-hr prepupae; qualitative and pronounced quantitative changes thus occur at the beginning of puparium formation.

Observations on fresh weight, crop condition, and spiracular morphology are presented, as these have proved of assistance in correlating the developmental progress of separate batches of larvae.

I. INTRODUCTION

The present account of developmental changes in certain constituents of larval haemolymph in the brown blowfly, *Calliphora stygia* (Fabr.) Schiner, arises from a study of gene action during development and metamorphosis. The investigation of haemolymph composition has been primarily directed towards an understanding of the regulation of gene activity in the larval fat body, and is expected to contribute to:

(1) identification of those genetic systems active in fat body (by detection of their products);

(2) distinction of replicative and transcriptive phases in cell development in this highly polytene tissue; and

(3) correlation of the changing patterns of gene activity with changes in hormone titres.

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Metamorphosis in holometabolous insects appears to involve re-utilization of at least peptides (Agrell 1964) or perhaps even proteins (Chen and Levenbook 1966*b*) synthesized during larval development. Dinamarca and Levenbook (1966) have suggested that possibly only 2% of the protein pool of the blowfly, *Phormia regina*, is newly synthesized during metamorphosis. Fundamental differences between the patterns of gene activity occurring before and after pupation are therefore to be expected.

The key role played by insect fat body in macromolecular synthesis and storage during larval life is well known (see review by Kilby 1963). The larval fat body has been identified as the site of synthesis of many of the haemolymph proteins in *C. erythrocephala* (Price 1966; Price and Bosman 1966). Investigations of other insects show the fat body to be the source of certain haemolymph lipids (e.g. Cook and Eddington 1967) and the principal centre of carbohydrate metabolism (Chefurka 1965; Wyatt 1967). Tissue uptake of haemolymph proteins has been demonstrated in both Diptera (Laufer and Nakase 1965) and Lepidoptera (Loughton and West 1965).

We report here on changes in the levels of proteins, lipids, and soluble carbohydrates in the haemolymph of third-instar larvae of *C. stygia*. As chronological age is not necessarily a sufficiently reproducible guide to developmental state, the observed trends in plasma composition have been correlated with changes in body weight, crop condition, and morphology of the spiracles.

II. Materials and Methods

(*a*) *Experimental Animals*

A laboratory strain of *C. stygia* was initiated from inseminated females collected at Melbourne, and maintained under mass-culture conditions for 2 yr. Small groups of larvae were separated immediately after hatching onto lean mammalian muscle on sawdust. The insectary temperature was maintained at approximately 21°C with a daily photoperiod of 16 hr.

(*b*) *Collection of Haemolymph*

After thorough washing to remove adhering debris, a known number of larvae were blotted dry on absorbent paper and weighed. The larvae were then chilled on ice, again dried, and bled through a small incision in the body wall into an ice-cold tube, then the haemolymph was processed as rapidly as possible. To avoid interference with chemical determinations, phenylthiourea (PTU) was used as an inhibitor of tyrosinase only in those cases specifically indicated.

Haemocytes were sedimented by centrifugation for 10 min at 2100 *g*. Aliquots of the cell-free supernatant (plasma), pooled daily from at least 50 larvae, were used for the estimations. Larvae from each age group were killed in boiling water for body length measurements.

(*c*) *Refractive Index*

The refractive index of haemolymph samples was determined using an Hitachi Perkin-Elmer hand protein refractometer, model PRP-B. Where necessary the plasma was diluted with an equal volume of saturated aqueous PTU solution to bring the refractive index within a convenient range. These determinations provided a relative measure of total dissolved solids in the haemolymph.

(*d*) *Protein Determinations*

The protein content of plasma aliquots was estimated by the Folin method of Lowry *et al.* (1951) modified by the use of citrate instead of tartrate (Eggstein and Kreutz 1955). In view of

criticism of the use of the Lowry method for measurement of insect blood protein (Pratt 1967), protein was precipitated from aliquots of plasma with 0·3N $HClO_4$, purified, taken up in 0·1N NaOH, and again estimated by the Lowry procedure. The purification procedure followed that outlined by Neufeld, Thomson, and Horn (1968) with the addition of the following step. The plasma protein, initially precipitated by $HClO_4$, was virtually all soluble in 95% ethanol and, in order to avoid loss during washing, protein was reprecipitated from the first wash of 95% ethanol by addition of an equal volume of 0·6N $HClO_4$. After precipitation from ethanol in this way, plasma proteins are no longer soluble in ethanol.

Inevitably some protein is lost during the repeated washings involved in purification. The average percentage change in weight of a series of accurately weighed samples of purified plasma protein taken through the washing steps a second time was used to correct for such losses. Protein concentrations were found by reference to standard solutions of purified bovine serum albumin and of purified haemolymph protein from *C. stygia*.

(*e*) *Amino Acid Analysis*

Purified, lyophilized plasma protein isolated from larvae at mid-instar 3 was dissolved in constant-boiling HCl, sealed *in vacuo*, and hydrolysed for 22 hr at 110°C. Analysis of the hydrolysate was carried out using a Beckman Spinco model 120B amino acid analyser.

(*f*) *Electrophoresis of Haemolymph Proteins*

Samples of plasma from bulk collections made at daily intervals throughout third instar were diluted to a protein concentration of approximately 20 mg/ml using 0·08M Tris–citrate buffer, pH 8·6. These samples were deep-frozen for subsequent electrophoretic examination on horizontal acrylamide (5%) gels. No appreciable change in protein mobilities was observed following such storage. Approximately 200 μg protein was loaded into each slot, and electrophoresis was carried out at 4°C using Poulik's (1957) discontinuous Tris–citrate/borate buffer system. Current was stabilized at 14 mA, while the voltage rose from 150 V initially to 250 V at the completion of the 3-hr run.

Gels were stained with amido black for detection of the proteins. Improved sensitivity was obtained by subsequent over-staining with Coomassie brilliant blue R250 (Chrambach *et al.* 1967).

(*g*) *Extraction and Estimation of Acid-soluble Carbohydrates*

Acid-soluble carbohydrate was extracted from plasma in three washes of ice-cold 0·3N $HClO_4$. The washes were combined, filtered through sintered glass, and deep-frozen for storage. Carbohydrate was determined colorimetrically by the anthrone method of Dimler *et al.* (1952) and values were expressed as glucose equivalents. Comparison of freshly prepared and frozen standard solutions of glucose showed that no change occurred during storage.

(*h*) *Lipid Determination*

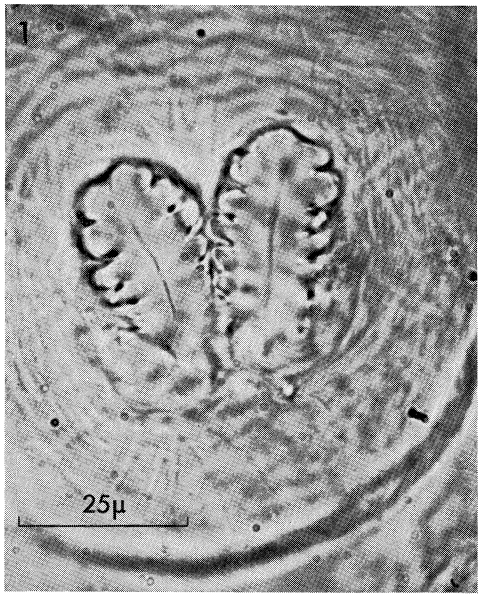
Lipid concentrations were estimated using Sudan black B. Aliquots of plasma were spotted onto Whatman No. 1 filter paper, dried, stained, and the colour subsequently eluted for photometric determination (Swahn 1952). A standard lipid extract prepared by the method of Folch, Lees, and Sloane-Stanley (1957) from an homogenate of larval fat body was used for reference.

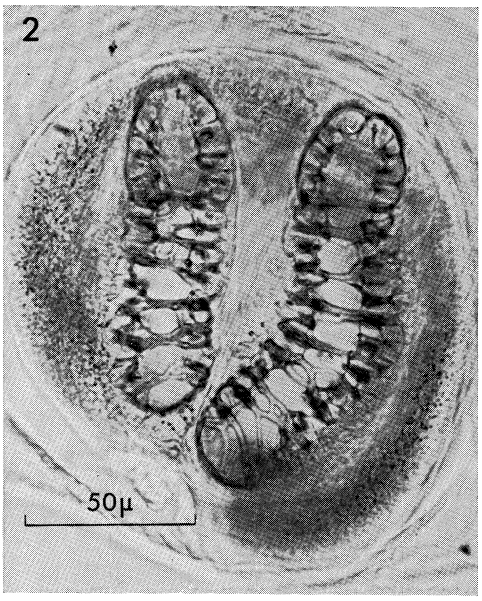
Staining of plasma lipids prior to electrophoresis was achieved by the addition of an equal volume of a solution of Sudan black B, a modification of the technique described by McDonald and Kissane (1960). Lipoproteins were separated by electrophoresis on acrylamide gel as for haemolymph proteins.

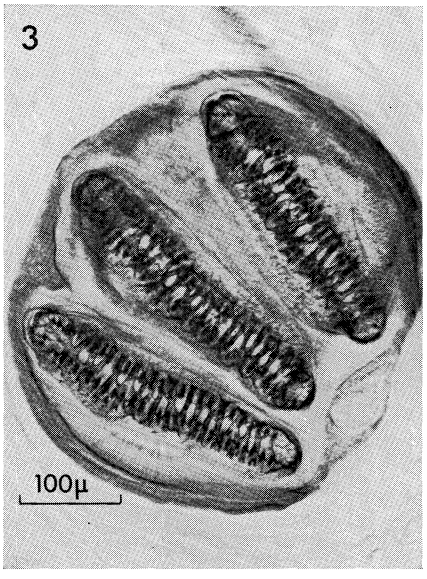
(*i*) *Estimation of Haemolymph Volume*

Individual larvae of known age, sorted into duplicate weighed groups of about 15, were injected with 2 μl of 2% azo rubin-S 180% (Sandoz) in insect saline (Ephrussi and Beadle 1936).

After 3 min, in which time the dye was found to be uniformly distributed throughout the haemocoele, each group of larvae was bled into an ice-cold tube containing a crystal of PTU. A few larvae, which showed excessive bleeding or in which the dye failed to become dispersed, were rejected. Following centrifugation to remove haemocytes, plasma samples of 100 μl were







Figs. 1–3.—Photomicrographs of cleared mounts of posterior spiracles from larvae of *C. stygia*. *1*, first instar; *2*, second instar; *3*, third instar after peritreme formation.

withdrawn from each tube and diluted to 3·5 ml with saturated aqueous PTU. The absorbance of each was then measured at 515 mμ against a blank containing 100 μl plasma from untreated larvae of the same age. Standard concentrations of the dye solution were used for reference to permit calculation of the mean haemolymph volume per larva from the observed dilution of the injected fluid.

III. Results

(*a*) *Larval Development*

Eggs of *C. stygia* are usually laid in groups of 50–250. One or two eggs in each group may hatch almost immediately after laying; hatching of the majority occurs synchronously about 18–24 hr later. The first and second larval moults occur after 1 and 3 days respectively, so that the larvae enter the third instar on day 4 of development. In the following account, "day 1" refers to the day of hatching.

The larval instars are easily recognized by observation of the posterior spiracles. During instar 1 (Fig. 1) each spiracle has two slits with simple marginal thickening. The two slits seen in instar 2 (Fig. 2) are larger with more heavily chitinized and complex borders. Three spiracular slits with complex margins are present at instar 3 (Fig. 3). The peritreme is incomplete until day 5 of development.

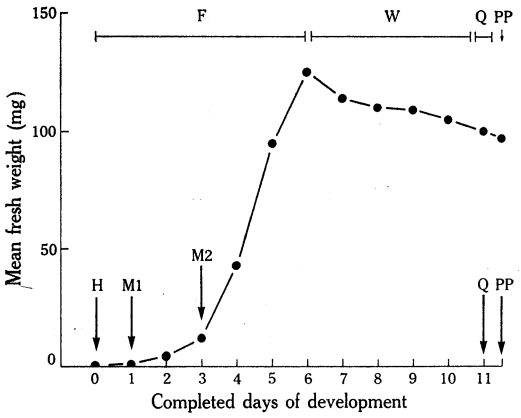


Fig. 4.—Daily fresh weights of individual larvae of *C. stygia*. Each point represents the mean for at least 50 larvae reared under standard conditions. *H*, hatching; *M1*, *M2*, first and second larval moults; *F*, feeding stage; *W*, wandering stage; *Q*, quiescent larval stage; *PP*, prepupa.

The *feeding stage* is followed at mid-instar 3 (day 7) by the *wandering stage*, when the larvae migrate to the sawdust and move about continuously. The later *quiescent stage* (day 11) is marked by inactivity and contraction of the body to assume at first a broad U-shape. The *prepupal stage* is reached when fully contracted, ovoid, quiescent larvae fail to become active when disturbed. By this time formation of a rigid puparium has commenced and tanning rapidly follows. The time required for development to this stage is affected by minor variations in culture conditions and especially by disturbance; prepupae normally form 11–12 days after hatching under the conditions used in this study.

The crop, readily visible through the body wall, changes during the final larval instar, and these changes have been found helpful in selecting larvae of uniform physiological condition. Early in this instar, the crop is a thin-walled sac containing liquefied food which reaches full distention as the larvae leave the meat. The food content of the crop is increasingly replaced by gas as digestion proceeds. In the late quiescent larva the crop collapses and its wall contracts.

The maximum rate of increase in fresh weight occurs early during instar 3 (Fig. 4). From the commencement of the last larval instar, length increases rapidly to 20 mm, this maximum being attained by day 6.

(*b*) *Haemolymph Volume*

Haemolymph volume reaches a maximum of 53 μl (Table 1) on day 6 and gradually falls to 43 μl in the quiescent larva at day 11. Table 1 also shows that the ratio of fresh weight to haemolymph volume remains constant during this period.

TABLE 1

DEVELOPMENTAL CHANGES IN WEIGHT AND HAEMOLYMPH VOLUME

Day	Larval Activity	(A) Mean Fresh Weight (mg)	(B) Mean Haemolymph Volume (μl)	A/B
4	Feeding	58	25	2·32
5	Feeding	99	42	2·36
6	Feeding	120	53	2·26
8	Wandering	108	48	2·25
9	Wandering	107	47	2·28
10	Wandering	104	44	2·36
11	Quiescent	101	43	2·35

Assuming that this ratio is still constant a little later in development, it is possible to estimate the haemolymph volume of the 3-hr prepupa as 42 μl. In this case a direct determination is not practicable due to inadequate mixing of haemolymph and injected dye.

(*c*) *Refractive Index and Protein Determination*

Plasma levels of total dissolved solids as estimated by refractometry show a rapid and almost linear increase in concentration from day 4 until a maximum level is reached at day 6. Thereafter there is a decline which is more marked at the transition of quiescent larvae to early prepupae (Fig. 5).

Protein determinations on whole plasma and on the purified precipitable plasma fractions by the Lowry method show changes paralleling those for total dissolved solids (Fig. 5). The values for purified protein were corrected to allow for an average weight loss during washing which was estimated experimentally at 10%. Purified haemolymph protein rather than bovine serum albumin was employed as a reference standard for the protein determinations, since use of the latter was found to cause underestimation of sample protein content by approximately 10%.

(*d*) *Amino Acid Analysis*

The average amino acid composition of purified, lyophilized plasma proteins from larvae at mid-instar 3 is shown in Table 2.

(*e*) *Electrophoretic Analysis*

Under the conditions used, 25 protein bands were regularly resolved in samples of larval, and 14 in prepupal haemolymph. These components all migrate towards the anode. Essentially the same pattern [Fig. 6(*b*)] is seen throughout the last larval

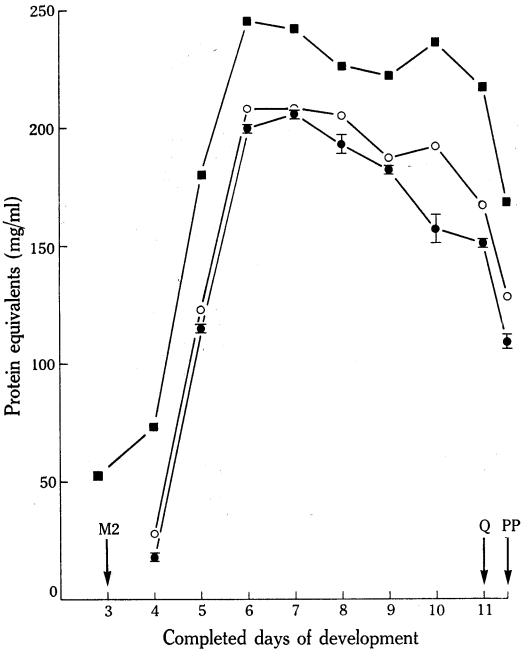


Fig. 5.—Changes in the concentrations of total dissolved solids, Lowry-positive material, and protein in pooled plasma from larvae of known ages. ■ Refractive index. O Lowry-positive material in whole plasma. ● Actual protein, determined by the Lowry method after purification. The vertical bars show the range of a minimum of two duplicate determinations. Where no ranges are indicated, duplicate determinations gave coincident values. *M2*, moult from second to third instar; *Q*, quiescent larvae; *PP*, prepupa.

instar, with the exception that from day 5 to day 6 there is a conspicuous increase in the relative concentration of one of four detectable lipoproteins, namely that represented by band 5.

TABLE 2

AMINO ACID ANALYSIS OF HAEMOLYMPH PROTEIN

Amino Acid	Concentration (μmoles/100 mg)	Amino Acid	Concentration (μmoles/100 mg)
Alanine	3·57	Lysine	8·15
Arginine	3·42	Methionine	3·53
Aspartic acid	12·23	Phenylalanine	10·40
Glutamic acid	10·28	Proline	3·92
Glycine	5·45	Serine	3·55
Histidine	3·06	Threonine	4·66
Leucine	6·79	Tyrosine	10·49
Isoleucine	4·45	Valine	6·06

Within 3 hr of puparium formation, pronounced changes occur in both the protein species present and in their relative concentrations [Figs. 6(*a*) and 6(*c*)].

Thus band 1 appears at the time of puparium formation, while bands 2, 24, and 25 increase in staining intensity. Loss or reduction of bands 3, 4, 6–8, 10, 12–14, 16, and 19 is evident at this stage. A decrease in the amount of protein present in bands 17, 18, 20, and 22 also occurs at this stage. Further, a change in mobility is shown by band 5, which migrates relatively faster after the onset of puparium formation.

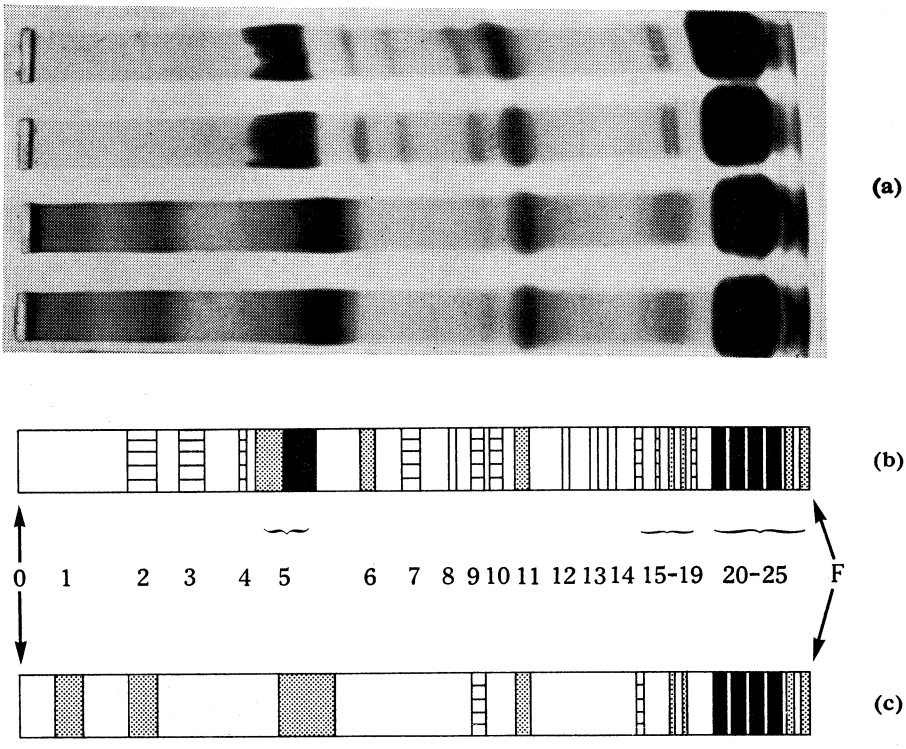


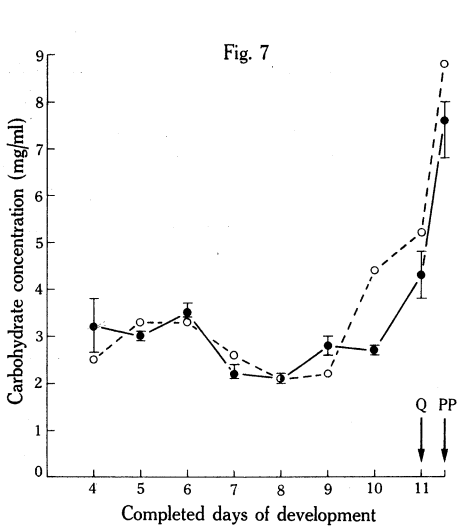
Fig. 6.—(*a*) Acrylamide gel separation of plasma proteins. The gel has been stained in amido black and overstained with Coomassie blue R250. The direction of migration is towards the anode, and the origin is marked by the slots formed in the gel. Upper two patterns: pooled plasma from 50 quiescent larvae; lower two patterns: pooled plasma from 50 prepupae less than 3 hr after puparium formation. (*b*), (*c*) Diagrams of acrylamide gel separations of plasma protein from quiescent larvae (*b*) and prepupae (*c*), showing the reference numbering system. The protein pattern of haemolymph from earlier instar-3 larvae is indistinguishable from that of quiescent larvae. The density of shading indicates staining intensities of the bands. Staining of larval haemolymph prior to electrophoresis showed that band 5 and three bands with mobilities close to those of 20–23 are lipoproteins. 0, origin; *F*, front.

(*f*) *Carbohydrate*

Plasma levels of acid-soluble carbohydrate (Fig. 7) drop slightly after day 6, reach a minimum level in mid-instar 3, and rise to a maximum in the prepupal stage.

(*g*) *Lipid*

Lipid levels in the larval plasma show a rapid and almost linear rise in concentration to day 6, and a further rise at day 9 (Fig. 8). There is a marked drop in the plasma lipid concentration in quiescent larvae and prepupae.



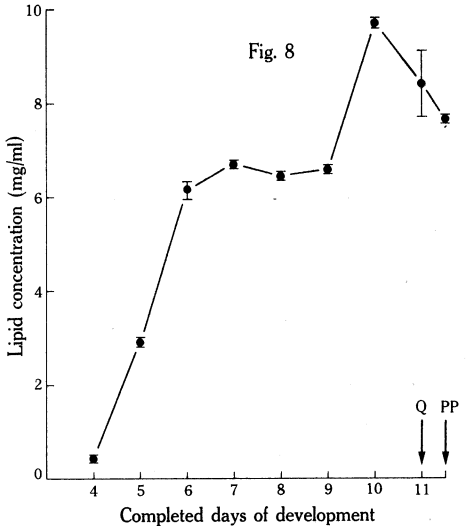


Fig. 7.—Concentration of soluble carbohydrates in larval plasma during third instar. ○, ● Determinations on separate batches of larvae. The vertical bars indicate the range of duplicate observations on one batch. *Q*, quiescent larvae; *PP*, prepupa.

Fig. 8.—Concentration of lipids in larval plasma during third instar. The vertical bars indicate the range of duplicate determinations. *Q*, quiescent larvae; *PP*, prepupa.

IV. Discussion

The outline of larval development of *C. stygia* presented here supplements earlier accounts (see particularly Fuller 1932; Mackerras 1933), and emphasizes those features which have proved of value in matching different batches of larvae according to the developmental stage reached rather than to their chronological age alone.

Larval blood volume was investigated to determine whether concentration changes in the plasma solutes reflect differences in the total amount of these components or simply result from changes in blood volume. The ratio of wet weight to haemolymph volume is in fact constant during the larval stage in *C. stygia*, as in *Phormia regina* (Chen and Levenbook 1966*a*).

Changes in the total dissolved solids of larval haemolymph are readily followed by monitoring refractive index as in mammalian plasma (Rubini and Wolf 1957). This method was reported by Martignoni and Milstead (1964) to provide useful estimates of total protein in insect plasma. During the larval development of *C. stygia*, changes in refractive index follow the same pattern as those shown by the concentration of protein estimated by the Lowry method. The contribution of

dissolved solids, other than proteins, to the refractive index of plasma therefore remains fairly constant over this period. Values for Lowry-positive material in aliquots of whole plasma correspond closely to those obtained for purified protein extracted from other aliquots at each stage. Gross interference with protein estimation by the Lowry method of the kind reported by Pratt (1967) in work on blood proteins of the adult cockroach, has not been encountered in this work with *C. stygia*.

A marked rise, both in protein concentration and in total protein, occurs in *C. stygia* between day 4 and day 6. There is approximately a 30-fold rise in total protein during this time. This increase coincides with a period of high synthetic activity in the fat body, which is accompanied by release of protein into the haemolymph (Martin *et al.*, unpublished data). Plasma proteins reach a maximum concentration of 200 mg/ml at days 6 and 7. Although this value is higher than that reported for most insects, it is close to the value found in *Phormia* (Chen and Levenbook 1966*a*). A decline in protein concentration occurs between mid-instar 3 (day 7), and the quiescent stage (day 11). During the transition from quiescent larva to prepupa, after day 11, this fall in protein concentration is very greatly accelerated. The drop in total plasma protein between days 7 and 11 is equivalent to a net withdrawal from the haemolymph of a calculated 4 mg of protein per larva. However, in the 12 hr preceding puparium formation, the decline is more abrupt, being equivalent to withdrawal of a further 2 mg per larva. The decline in protein level from mid-instar 3 is probably due to some uptake by tissues and to some metabolic utilization of protein at a time when the rate of protein synthesis is greatly reduced. Close to puparium formation, tissue uptake of plasma proteins is markedly enhanced in Lepidoptera (Loughton and West 1965) and is thought to occur also in *Phormia* (Chen and Levenbook 1966*b*). A decline in protein synthesis towards the end of instar 3 has been observed when fat body from larvae of *C. erythrocephala* is incubated *in vitro* in a suitable medium (Price 1966). Protein synthesis *in vivo* increases slightly just before pupation (Wimer, cited by Wimer and Lumb 1967) or at the onset of pupation (Chen and Levenbook 1966*b*).

The drop in both protein concentration and total protein from mid-instar 3 to day 11 is apparently non-selective, as electrophoretic patterns of plasma proteins are essentially identical during this period. Electrophoretic studies of the plasma proteins of prepupae show that the observed drop in both protein concentration and in total protein, during the transition from quiescent larvae to prepupae, represents a loss or disproportionate decrease in certain protein species. This is consistent with either selective uptake by the tissues or selective degradation. This contrasts sharply with the apparently non-selective fall which occurs between mid-instar 3 and the quiescent stage. However, the appearance of at least one new plasma protein [band 1, Fig. 6(*c*)] in prepupae provides evidence that synthesis is occurring at this period. Electrophoretic separations were performed in each case on replicate samples of bulk plasma pooled from a minimum of 50 larvae of a laboratory strain which had undergone some inbreeding. It is therefore unlikely that individual variations in plasma protein could have contributed to the apparent differences between age groups.

No detectable differential changes in the relative concentrations of particular protein species are observed between day 6 and the commencement of puparium

formation. For this reason, the average amino acid composition of bulk plasma protein would be expected to remain approximately constant during this period.

The anthrone method is in general use for the quantitative estimation of glucose derivatives from both reducing and non-reducing carbohydrates. This procedure may lead to estimates which are somewhat high due to interference by non-carbohydrate, anthrone-positive material (Orr 1964). Tryptophan, which is known to be anthrone positive, is present in the larval haemolymph of *C. augur* in negligible amounts (Hackman 1956).

Although the *concentration* of acid-soluble carbohydrate remains essentially constant during days 4–6 of development, each larva shows a threefold increase in the *total* acid-soluble carbohydrate in the plasma, when the change in haemolymph volume occurring in this period is considered. A definite and reproducible fall in the concentration of acid-soluble carbohydrate, and in the total amount per larva, takes place in mid-instar 3, with a marked rise in both at the end of the instar. No consideration has been given in the present study to the bound carbohydrates of the blood which may comprise a significant proportion of the total blood carbohydrates as in, for example, *Periplaneta* (Lipke, Grainger, and Siakotos 1965).

The significance of developmental changes in the levels of acid-soluble carbohydrate is difficult to determine, for this "free" pool is closely interrelated with the bound material in the blood, with storage carbohydrates in the tissues (particularly in the fat body), and with the structural carbohydrates of the cuticle (for review see Wyatt 1967). Changing metabolic demands (Crompton and Birt 1967) and humoral agents (Wiens and Gilbert 1967), particularly at the time of pupation, are likely to be especially important.

The insect fat body has been identified as the site of synthesis of neutral haemolymph lipids (Cook and Eddington 1967; Wlodawer and Lagwinska 1967) and of haemolymph phospholipids (Thomas and Gilbert 1967). Conjugation of haemolymph lipids with carrier proteins has been widely demonstrated (Siakotos 1960; Adiyodi and Nayar 1967). Plasma lipid levels in insects are known to vary with changing metabolic demands and to be subject to hormonal control (Gilbert 1967).

In *C. stygia*, larval plasma at mid-instar 3 shows 4 lipoprotein bands separable by electrophoresis. The lipoprotein component showing lowest mobility [band 5, Fig. 6(*b*)] is quantitatively the most significant. It is detectable throughout the instar, but shows a sharp increase from day 5 to day 6.

There are no marked changes in the species of haemolymph protein which are synthesized between the commencement of instar 3 and the onset of pupation. The plasma protein concentration rises abruptly between days 3 and 6 of development, thereafter decreasing until the formation of the puparium. The appearance of a new protein species in the haemolymph of the early prepupa apparently reflects a different pattern of gene activity. Trends in the concentration of both soluble carbohydrate and lipid alter at day 6 and again at day 10, or just after. In the case of the plasma protein and carbohydrate levels, the trends established at about day 10 are greatly accelerated at the time of puparium formation. The synthesis and release of haemolymph proteins at the stages marked by the apparent discontinuities in development during the third instar will be discussed in a subsequent paper in this series.

V. Acknowledgments

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