

DEVELOPMENTAL CHANGES IN THE LATE LARVA OF *CALLIPHORA STYGIA*

IV.* UPTAKE OF PLASMA PROTEIN BY THE FAT BODY

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Abstract

The protein content of the larval fat body of *C. stygia* increases during late third-instar development from 1.0 mg at day 7 to 3.4 mg in the white prepupa (day 11). This increase is due predominantly to uptake of protein from the haemolymph, in which there is a concurrent fall in protein concentration. The rate of uptake rises from 0.6 mg/day at days 8–9 to 0.9 mg/day at days 10–11.

Five plasma proteins (designated H15–19) appear to be taken up differentially by the fat body *in vivo* and *in vitro*. The uptake of these species occurs without degradation to constituent amino acids, but involves conformational changes which reverse those occurring earlier in the instar when protein is released into the haemolymph from the fat body.

I. INTRODUCTION

The role of the fat body during larval development in holometabolous insects is remarkably complex, involving many aspects of protein, lipid, and carbohydrate metabolism (Kilby 1963). The larval fat body of *Calliphora stygia* undergoes, during third-instar development, a series of sharply defined changes in function relative to protein synthesis and release:

- (1) a period of very active protein synthesis in the fat body, with rapid concurrent release into the haemolymph;
- (2) abrupt cessation of the synthesis by the fat body of many protein species, including those constituting some 70% of the total haemolymph protein (Kinnear, Martin, and Thomson 1971);
- (3) uptake of specific proteins from the haemolymph into fat body cells, measured directly in the present work and correlated with decreasing haemolymph protein concentrations and increasing protein content of the fat body late in larval life;
- (4) a phase of increased protein synthesis, with retention of the nascent protein, close to puparium formation (Martin, Kinnear, and Thomson 1969).

The haemolymph thus serves as a temporary storage organ for protein until the predominant function of the fat body changes from synthesis to storage. The ultimate objective of the present study of the interaction between these tissues is the elucidation of the genetic control of each step in the chain of events involving synthesis,

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temporary storage, cellular uptake, sequestration, mobilization, and finally re-use of specific proteins. Clearly this is a key system in the development of adult tissues during metamorphosis in holometabolous insects.

II. MATERIALS AND METHODS

(a) *Experimental Animals*

The strain of *C. stygia* used, and the culture conditions, have been described previously (Kinnear *et al.* 1968).

(b) *Radioisotopes*

A mixture of uniformly ^{14}C -labelled amino acids (specific activity 52 mCi/m-atom), purified by chromatography from an hydrolysate of *Chlorella* proteins, was obtained from the U.K.A.E.A. Radiochemical Centre, Amersham, England.

(c) *Preparation of Isotopically Labelled Haemolymph Proteins*

Day 5 (feeding) larvae (Kinnear *et al.* 1968) each received $1.5 \mu\text{Ci } ^{14}\text{C}$ -amino acid mixture in $1.5 \mu\text{l}$ water. The larvae were incubated for 90 min at 25°C before bleeding. Haemolymph from groups of 50 larvae was collected at 0°C , using a trace of phenylthiourea to inhibit phenol oxidase activity. Plasma was dialysed for 24 hr at 4°C against three changes of saline (Ephrussi and Beadle 1936) containing phenylthiourea. The product contained $57 \mu\text{g}$ protein/ μl and 3770 counts/min/ μl . Between 97 and 98% of this radioactivity was precipitable with perchloric acid.

(d) *Uptake of Plasma Proteins in vivo*

The development of wandering (day 8) and late wandering (day 10) larvae was synchronized by wet storage (Thomson, Imray, and Horn 1970). Three groups of 10 larvae of each age (wandering: A, B, and C; late wandering: D, E, and F) were treated. Each larva was injected with $3 \mu\text{l}$ ^{14}C -plasma protein solution. Groups A and D were incubated for 5 min; the remaining groups were incubated at 25°C , in dry sawdust. When dissected after 24 hr, groups B and C were still at the wandering stage while groups E and F comprised white prepupae.

(e) *Uptake of Plasma Proteins in vitro*

A concentrated supplement ($\frac{1}{8}$ vol.) of amino acids and salts was added to dialysed ^{14}C -labelled plasma proteins, to achieve the final concentrations given by Price (1969) and Robb (1969), at pH 7.1. Replicate samples of fat body from white prepupae were incubated in this medium for 2.75 hr at 25°C .

(f) *Examination of Proteins*

Plasma, incubation medium, and tissue proteins were prepared for liquid scintillation counting (Neufeld, Thomson, and Horn 1968) and electrophoresis. Electrophoresis was carried out at 4°C on horizontal 5% acrylamide gel using a discontinuous Tris-citrate/borate buffer system at pH 8.7/pH 8.2 (Kinnear, Martin, and Thomson 1971). Proteins were stained with amido black and the gels were subsequently dried and autoradiographed against X-ray film. Other separations of plasma and fat body proteins were carried out in vertical 5% acrylamide gels using continuous Tris-borate-EDTA buffer systems (Raymond 1964) at pH 8.2 and 9.4. For comparison of molecular sizes, additional samples were separated to equilibrium (20 hr) on vertical-gradient acrylamide gels (4–20% Gradipore; Townson and Mercer Pty. Ltd., Australia) using 10 mA, 80 V at 2.5°C .

III. RESULTS

(a) *Quantitative Determination of Uptake in vivo and in vitro*

The distribution of radioactivity in plasma and in fat body of recipient larvae, 24 hr after injection of labelled haemolymph protein, is shown in Table 1. At both the

developmental stages considered, uptake of plasma protein by the fat body apparently takes place. About 10% of the initial radioactivity in the plasma was found in the fat body at the end of a 24-hr period covering the transition from the wandering to the late wandering stage. After an incubation period of similar duration, the fat body of a late wandering larva developing to the prepupal stage had taken up about 20% of the isotope injected.

Approximately 2.8% of the radioactivity administered to the larvae was initially in an acid-soluble form. After the 24-hr incubation from days 8-9 (groups B and C, Table 1), the total counts in acid-soluble material remained in the range 2.8-3.8%. After 24 hr incubation from days 10-11 (groups E and F, Table 1), the proportion of radioactivity in the acid-soluble fractions from haemolymph and fat body was 6.8-7.6%. The mean proportion of the injected radioactivity recovered in haemolymph and fat body together (excluding other tissues) was 94% for groups B and C, and 92% for groups E and F (data of Table 1).

TABLE 1
DISTRIBUTION OF RADIOACTIVITY (COUNTS/MIN/LARVA) IN HAEMOLYMPH
AND FAT BODY 24 HR AFTER INJECTION OF ^{14}C -LABELLED PLASMA
PROTEIN

Duplicate determinations					
Group	(1) Haemolymph		(2) Fat Body		Total for (1) and (2)
	Acid- insoluble	Acid- soluble	Acid- insoluble	Acid- soluble	
B (days 8-9)	9500	200	900	100	10700
C (days 8-9)	9000	300	1100	100	10500
E (days 10-11)	7700	500	2000	300	10500
F (days 10-11)	7300	500	2300	200	10300

The haemolymph volume of larvae from groups A (day 8) and D (day 10) may be estimated from the dilution of the injected isotope. The incubation time allowed (5 min) was sufficient for adequate mixing with endogenous haemolymph (Kinnear *et al.* 1968), without measurable loss by tissue uptake. The haemolymph volume of larvae of group A was approximately 45 μl , and that of batch D approximately 35 μl . The total haemolymph protein per larva was thus 6.6 and 4.5 mg for animals of groups A and D respectively. Since the amount of uptake of the labelled fraction of the haemolymph over 24 hr is also known (Table 1), the total protein uptake of the fat body of each larva may be calculated (Table 2). Determinations of the total fat body protein for larvae of day 9 (late wandering) and day 11 (white prepupae) are shown in Table 2 for comparison.

Fat body from animals at the stage of puparium formation also showed marked uptake of ^{14}C -labelled haemolymph protein when incubated *in vitro* for 2.75 hr. At the end of this period, replicate incubations yielded tissue proteins with specific activities of 3000–3500 counts/min/mg.

TABLE 2
PROTEIN UPTAKE AND TOTAL PROTEIN
PER FAT BODY
Duplicate determinations

Group	Total Protein (mg)	Protein Uptake over 24 hr (mg)
B	2.1 (day 9)	0.54 (days 8–9)
C	2.1 (day 9)	0.66 (days 8–9)
E	3.4 (day 11)*	0.82 (days 10–11)
F	3.4 (day 11)*	0.94 (days 10–11)

* White puparia.

(b) *Protein Species Involved in Uptake by Fat Body*

Qualitative comparisons of the ^{14}C -labelled proteins of plasma and fat body extracts from recipient larvae are presented in Figures 1 and 2. The spectrum of plasma proteins of larvae from each group is shown in the acrylamide-gel separation stained with amido black [Fig. 1(a)], while an autoradiograph of the same gel is presented to show the labelled species present [Fig. 1(b)]. The bulk of the radioactivity received by recipient larvae was located in those plasma bands designated H15–19 and, to a much lesser extent, in bands H4, 10, 11, 20, and 21 (for details of band numbers see Kinnear, Martin, and Thomson 1971).

An electrophoretic separation of the soluble proteins of fat body from recipient larvae is shown in Figure 2(a). An autoradiograph of this gel [Fig. 2(b)] revealed that radioactivity was present in fat body bands F15–19. These are electrophoretically comparable with plasma proteins H15–19, except that the fat body contains no band corresponding to the slower of the two overlapping components of H15 (Kinnear, Martin, and Thomson 1971). There was no apparent radioactivity at the positions of bands H4, 11, 20, or 21.

The separation of soluble proteins from fat body incubated *in vitro* with radioactive plasma protein showed a pattern of bands identical with that of freshly dissected tissue. The bulk of the radioactivity taken up by the fat body was again associated with bands F15–19.

(c) *Relationship of Plasma and Fat Body Proteins*

A contrast in the rate of migration of the predominant components of plasma and fat body is evident in separations on 5% acrylamide gels at pH 8.2. At this pH,

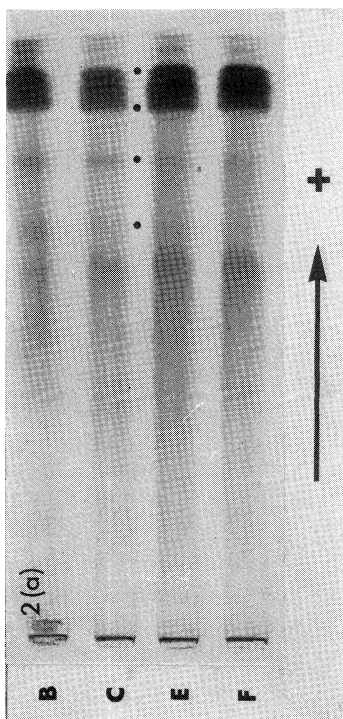
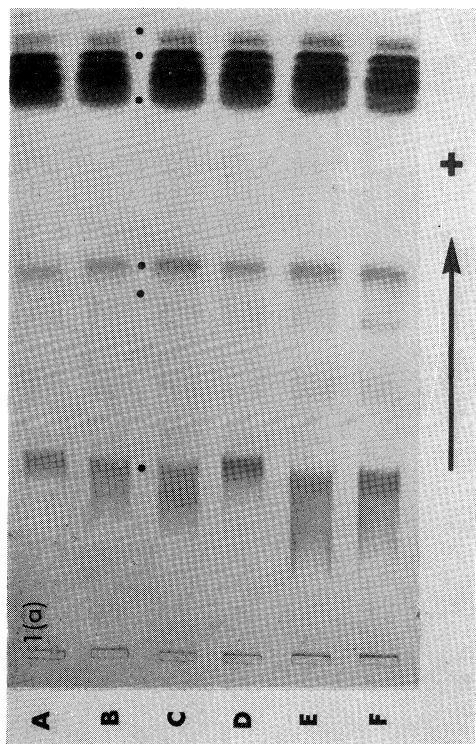
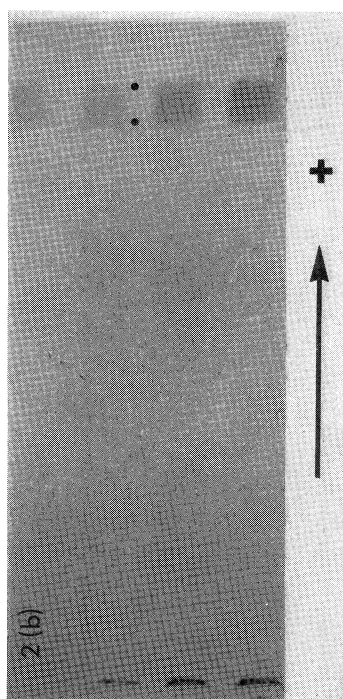
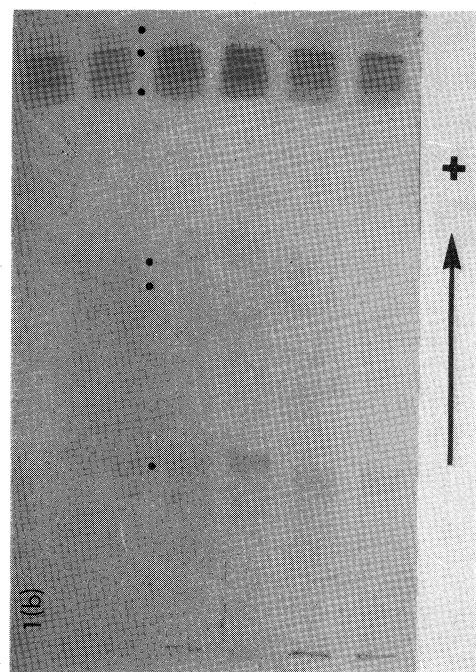


Fig. 1.—(a) Electrophoretic separation of plasma proteins 5 min (A, D) or 24 hr (B, C, E, F) after injection of ^{14}C -plasma proteins into wandering (A-C) or late wandering (D-F) larvae. Bands indicated $\bullet$ are, from left to right: H4, 10, 11, 15, 19, and 21. (b) Autoradiograph of gel shown in (a); exposure 6 weeks. Bands marked $\bullet$ are H4, 10, 11, 15, 19, and 21.

Fig. 2.—(a) Electrophoretic separation of fat body proteins 24 hr after injection of ^{14}C -plasma proteins into wandering (B and C) or late wandering (E and F) larvae. Bands indicated $\bullet$ are, from left to right: F10, 13, 15, and 19. (b) Autoradiograph of gel shown in (a); exposure 6 weeks. Bands marked $\bullet$ are F15 and 19.

the bulk of the plasma protein moves as a single zone close to the middle of the separation ($R_m = 0.6$), whereas the predominant proteins of the fat body migrate closer to the front (Fig. 3).

Electrophoresis to equilibrium on gradient acrylamide gels shows that the predominant proteins of fat body and plasma are similar in molecular size at pH 9.4 (Fig. 4). Such separations also reveal that fat body homogenates contain additional low molecular weight constituents.

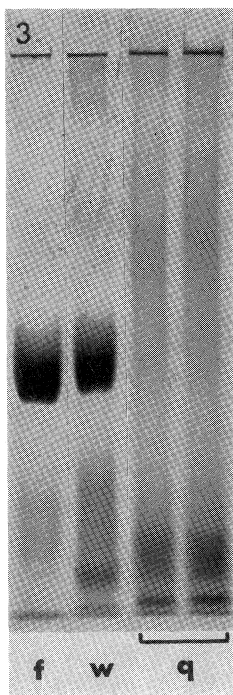


Fig. 3.—Electrophoretic separation of plasma and fat body proteins in a continuous buffer system at pH 8.2. Plasma from feeding (*f*) and wandering (*w*) larvae. Fat body from quiescent (*q*) larvae.

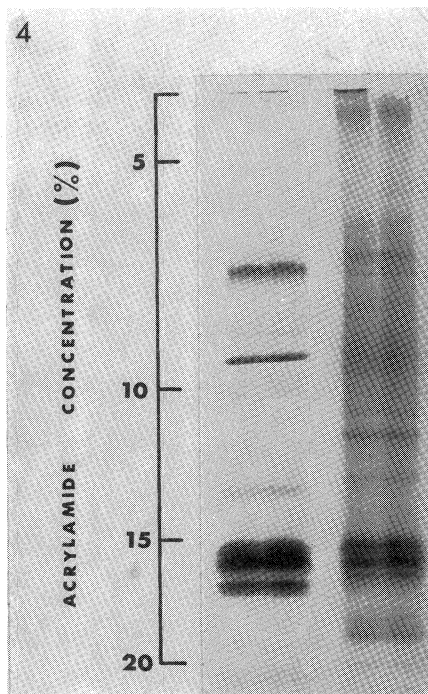


Fig. 4.—Electrophoretic separation of plasma and fat body proteins on a gradient gel of acrylamide using continuous buffer system at pH 9.4. Two plasma samples on left; fat body on right.

IV. DISCUSSION

After the commencement of the wandering phase of larval development at day 7, the fat body of *C. stygia* shows a major, progressive increase in protein content up to the prepupal stage (Table 2, and see also Table 1 in Martin, Kinnear, and Thomson 1969). Concurrently, synthetic activity in the fat body is greatly reduced, especially from day 7 to day 10. Circumstantial evidence that the fat body sequesters protein from the haemolymph is provided by an accompanying fall in plasma protein concentration (Kinnear *et al.* 1968). Further, the time course of the changes in plasma protein concentration suggests that withdrawal of protein from the haemolymph is accelerated in the last 12 hr before puparium formation.

The present analysis provides direct evidence of uptake of haemolymph proteins by fat body cells both *in vivo* and *in vitro*. The uptake over a 24-hr interval *in vivo* is greater over days 10–11 of development than over days 8–9 (Table 2). In insects of both ages, 92–94% of the injected radioactivity is recovered from fat body and haemolymph alone after 24 hr, so that uptake of haemolymph protein, or utilization of its catabolites, by other tissues must be slight during larval life. Nevertheless, the uptake of haemolymph protein by cells of the salivary gland is measurable at this stage (M.-D. Martin, J. F. Kinnear, and J. A. Thomson, unpublished data). Degradation of labelled haemolymph protein appears to be greater in older larvae injected close to puparium formation, as a higher proportion of the radioactivity is then found in the acid-soluble fraction of the fat body. In general, especially at the wandering stage, exogenous haemolymph protein appears to be maintained in a high molecular weight form during and after uptake and sequestration. Isolated fat bodies from wandering larvae also take up ^{14}C -labelled plasma proteins from the incubation medium, again with no evidence of marked degradation.

The total fat body protein at day 7 is approximately 1.0 mg/larva (Martin, Kinnear, and Thomson 1969). If it is assumed that uptake of plasma proteins commences at day 7 and continues steadily (see Fig. 5 in Kinnear *et al.* 1968) at about 0.6 mg/fat body/24 hr to day 10, then rises to 0.9 mg for the last 24 hr before puparium formation (Table 2), the protein content of the early prepupal stage would be 3.7 mg/fat body. While this value should clearly be regarded as an approximation, it is in good agreement with the observed value (3.4 mg/fat body, Table 2) for animals of this age. The estimated haemolymph volume for day 10 larvae, on which certain estimates of Table 2 are based, is lower than that given by Kinnear *et al.* (1968) owing to the smaller size of larvae in this particular group. The factor responsible for this difference is the loss of body weight resulting from the short-term wet storage used here to ensure synchronization of development.

The bulk of the increase in fat body protein which occurs between days 7 and 11 of larval development is due to an increase in the proteins of bands F15–19, and, at the quiescent stage, to bands F4–10 also (Kinnear, Martin, and Thomson 1971). The fat body protein spectrum of third-instar larvae is qualitatively similar (Kinnear, Martin, and Thomson 1971) during the period from day 4 to day 11, which includes both feeding and wandering stages. In the feeding larvae, release of fat body proteins to the plasma is a major process. Conversely in late wandering to quiescent larvae, uptake of plasma proteins into the fat body exceeds release. Fat body proteins F15–19 of feeding larvae are synthesized in this tissue. These species are also represented as bands F15–19 of wandering and quiescent larvae where they are predominantly derived by uptake from the plasma. The autoradiographic data show that isotopically labelled proteins corresponding to fractions H15–19 of the plasma appear in the fat body in appreciable amounts after incubation either *in vivo* (Fig. 2) or *in vitro*. After uptake, these protein species must remain at least largely soluble in the buffer used for extraction. Homology between proteins F15–19 and H15–19 is confirmed by the similarity in molecular sizes revealed on gradient-gel electrophoresis (Fig. 4). Nevertheless these species are not identical. A reversible change, presumably in conformation, occurs both on release of these components from the fat body to the haemolymph, and on their subsequent uptake. Evidence of such an alteration is seen

in the association of species H15-19, but not F15-19, into a single zone at pH 8.2 (Fig. 3). Native plasma proteins form soluble coacervates in ethanol (Kinnear *et al.* 1968), whereas the corresponding fat body proteins are insoluble in alcohol. Further, plasma bands H15-19 include some lipoproteins (Kinnear *et al.* 1968, bands 20-23) but there is no detectable lipid associated with fat body bands F15-19.

There is no evidence of uptake of the proteins represented by bands H4, 10, 11, 20, and 21. These species would be detected in long-exposure autoradiographs if they were not either differentially excluded during uptake, or substantially altered (as, for instance, by conversion to insoluble storage materials).

Active synthesis of proteins corresponding to bands F6-10, 19, and 22 takes place in the fat body of wandering and quiescent larvae (Kinnear, Martin, and Thomson 1971). With the exception of band F19, there is no detectable label in these regions when fat body proteins are examined 24 hr after injection of radioactive plasma proteins. While synthesis of F19 occurs in these animals, it cannot involve amino acids derived from the breakdown of labelled larval plasma protein as, in this case, label should also appear in the proteins F6-10 and F22. The label which does appear in F19 must arise from the accumulation of the homologous plasma protein. It follows that neither the small percentage of radioactivity administered in acid-soluble form (less than 3% of the injected ^{14}C) nor low molecular weight products derived from the labelled protein makes a significant contribution to the precursor pool from which *de novo* protein synthesis occurs in the fat body. This result confirms that exogenous plasma proteins are conserved in high molecular weight form before and after uptake.

Uptake of haemolymph protein has now been proposed as one source of the protein reserves of the late larval and pupal fat body in a number of holometabolous insects (Locke and Collins 1968). Locke and Collins (1965, 1966, 1968) demonstrated that both sequestration of haemolymph protein and lysis of cytoplasmic organelles contribute to the development of protein granules in *Calpodes*. Exogenous proteins, such as horseradish peroxidases, were taken up from the haemolymph into the granules at a time when the blood protein level was decreasing. In studies of other Lepidoptera, the fat bodies of *Malacosoma* (Loughton and West 1965), *Pieris* (Chippendale and Kilby 1969), and *Diatraea* (Chippendale 1970) have been shown to absorb haemolymph proteins selectively at pupation.

The mechanism by which cells take up intact proteins, and the control of this process now require elucidation (Ryser 1968). In *Calliphora*, storage of haemolymph protein in the fat body commences considerably earlier than the rise in the level of endogenous moulting hormone (Shaaya and Karlson 1965) which precedes puparium formation. Temporal separation of the principal period of synthetic activity from uptake and sequestration suggests sequential, perhaps nucleus-mediated, control of these events. There is direct evidence in *C. stygia* of changing nuclear activity between the periods characterized respectively by synthesis and by uptake of protein in the fat body. This evidence includes sharply defined changes in (a) the morphology of the polytene chromosomes (Thomson 1969); (b) the nature and amount of nuclear-inclusion ribonucleoprotein (Thomson and Gunson 1970); (c) the structure of the nuclear-cytoplasmic boundary (Thomson, Rogers, Gunson, and Horn 1970); and (d) the pattern of nuclear binding of exogenous moulting hormone (Thomson, Rogers, Gunson, and Horn 1970).

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