DEVELOPMENTAL CHANGES IN THE LATE LARVA OF *CALLIPHORA STYGIA*

II.* PROTEIN SYNTHESIS

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Summary

The pattern of incorporation of [^{3}H]leucine into proteins of fat body, plasma, body wall, and salivary gland has been studied in third-instar larvae of *C. stygia*. The rate of incorporation is higher in these tissues at day 5 of development than at days 7 and 11 (quiescent stage).

The amount of acid-precipitable protein in the fat body increases little between days 5 and 7 in spite of the high synthetic rate early in the instar, whereas from day 7 to 11 there is almost a threefold increase. Simultaneously, the protein content of the plasma increases fourfold from day 5 to 7, and decreases by approximately one-third from day 7 to 11. The total protein of the body wall tissues remains constant from day 5 on, whilst that of the salivary gland cells shows a slow and steady increase during the instar.

Changes in the relative specific activities of fat body and plasma proteins with age suggest two major alterations in the biosynthetic functions of fat body: (1) the cessation, between days 5 and 7, of the phase of intense protein synthesis associated with rising levels of plasma protein; (2) the initiation, at the quiescent stage, of a new phase of synthesis involving protein retention in the fat body. In addition, the reduced level of plasma protein between days 7 and 11 apparently reflects uptake by the fat body at this time. Protein synthesis in the tissues of the body wall decreases after day 5, and there is no evidence of any subsequent major change in protein biosynthesis in this tissue. Salivary gland cells appear to show a transition from rapid protein synthesis and quick release of protein at day 5, to slower synthesis with increased protein storage at the quiescent stage.

I. INTRODUCTION

Apart from the primary question of the nature and maintenance of determination, the qualitative and quantitative regulation of tissue-specific proteins in higher organisms appears as the central problem of morphogenesis. In holometabolous insects, certain larval tissues which may already be recognizably distinct with regard to cell morphology, undergo further differentiation at specific developmental stages. This differentiation is characterized by abrupt changes in cell metabolism. Such pronounced discontinuities in cell function suggest the coordinate switching, probably mediated by morphogenetic hormones, of several, or perhaps in some cases many, genetic units.

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The present report deals with the temporal pattern *in vivo* of gross protein synthesis in fat body, body wall, salivary gland, and plasma in third-instar larvae of the brown blowfly, *Calliphora stygia* (Fabr.) Schiner. These tissues differ markedly in structure, function, and fate at metamorphosis. The period under study covers the portion of the life cycle from day 5, when the larva is actively feeding and body weight is increasing most rapidly, to the onset of puparium formation at day 11, when body weight is falling (Kinnear *et al.* 1968), and the insect has become, except for respiration, a closed system for the period of imaginal development. Major changes in food intake and utilization, in plasma protein, lipid, and carbohydrate levels, and also in behaviour (Kinnear *et al.* 1968) take place during this time. Coordinated activation or deactivation of groups of genes could therefore be expected during this phase of development, and should be reflected in changing patterns of protein synthesis in the various tissues. The rising titre of the endogenous moulting hormones associated with puparium formation (Shaaya and Karlson 1965) may be implicated more or less directly in the regulation of gene activity close to pupation. The key problems of the mechanisms involved in specific tissue function and in selective response of "target" tissues to the action of morphogenetic hormones will become more accessible to analysis only when the critical phases of genetic read-out have been delimited for each developmental stage.

The time-course of synthesis of certain specific proteins in each tissue will be discussed elsewhere (Kinnear, Martin, and Thomson, unpublished results), while a study of uptake of plasma proteins by tissues of the late larva and prepupa will form the basis of a subsequent paper in this series.

II. Materials and Methods

(*a*) *Experimental Animals*

Larvae of *C. stygia* from the laboratory strain previously described (Kinnear *et al.* 1968) were reared in small groups on excess lean mammalian muscle at an insectary temperature of approximately 21°C and a daily photoperiod of 16 hr.

(*b*) *Chemicals*

L-[4,5-^{3}H]leucine, with a specific activity of 23·5 or 14·7 Ci/m-mole, was obtained from the Radiochemical Centre, Amersham, England. All other chemicals used were of analytical reagent grade.

(*c*) *Procedure for Incorporation Studies*

Day-5 (feeding), day-7 (wandering), and day-11 (quiescent) larvae were selected for the experimental work from groups of animals which showed typical developmental progress using the criteria of body weight, haemolymph refractive index, crop condition, and behaviour (Kinnear *et al.* 1968). Larvae were anaesthetized on ice and isotopically labelled amino acid [2 μCi in 2 μl ($8 \cdot 5 \times 10^{-8}$ mM) or 1·5 μCi in 3 μl ($10 \cdot 2 \times 10^{-8}$ mM)] was injected into the haemocoele as described by Neufeld, Thomson, and Horn (1968). Incorporation times of 5, 10, 30, 90, 240, 360, or 480 min were allowed. The larvae were incubated during this period at 25°C in moist cellulose tissue, on which any loss of haemolymph could be readily detected by its melanization. Larvae which bled from the injection site were rejected. All comparisons were based on a minimum of two, but usually three, groups of five larvae.

After the appropriate incubation, haemolymph was collected into an ice-cold tube through a small incision in the body wall, and dissections were performed rapidly in a large volume of ice-cold insect saline (Ephrussi and Beadle 1936). Sections of body wall were turned inside out

to facilitate washing, and tissues, other than epidermis with some attached muscle, were removed. The fat bodies were dissected free of malpighian tubules and other adherent organs. Fat tissue at the posterior end of the salivary glands was rejected, and the glands were then cut into small sections, opened out, and scrupulously washed in saline. Two additional washes in ice-cold saline preceded further treatment of all tissues.

(*d*) *Preparation of Tissue Proteins*

Haemolymph was centrifuged for 10 min at 2000 *g* to sediment haemocytes. Plasma protein was then precipitated and purified from aliquots of the cell-free supernatant as outlined by Kinnear *et al.* (1968). Washed fat body, body wall, and salivary gland were homogenized in cold 0·3N $HClO_4$ and, in the case of body wall preparations, pieces of cuticle were removed by hand. Purification of the acid-insoluble proteins from each tissue followed the procedure of Neufeld, Thomson, and Horn (1968); this treatment effectively removed unincorporated isotope. The washed, dry protein was taken up in a known volume of 0·1N NaOH.

(*e*) *Estimation of Tissue Proteins*

The protein content of aliquots from the NaOH solutions of tissue residues was estimated by the Folin method of Lowry *et al.* (1951), modified by the use of citrate instead of tartrate (Eggstein and Kreutz 1955). Purified bovine serum albumin was employed as a reference standard for the protein determination.

(*f*) *Measurement of Radioactivity*

Aliquots of the protein solutions were absorbed onto discs of Whatman GF/C glass fibre in counting vials and were dried overnight at 40°C. Toluene-based scintillation fluid was then added and the radioactivity measured using a Nuclear-Chicago model 6850 scintillation spectrometer. An average counting efficiency of 14% was obtained. Correction was made for background activity. Results have been expressed as specific activities unless otherwise indicated.

III. Results

The time-course of incorporation of [^{3}H]leucine into the fat body, body wall, salivary gland, and plasma protein of day-5, day-7, and day-11 larvae is shown in Figure 1 for specified intervals up to 240 min after injection. In day-5 larvae, fat body, body wall, and salivary gland proteins showed rapid incorporation of the

Table 1

DEVELOPMENTAL CHANGES IN TISSUE PROTEIN CONTENT

Tissue	Estimated Protein Content (mg/larva)		
	Day 5	Day 7	Day 11*
Plasma	2·5	10·0	6·5
Fat body	0·8	1·0	2·8
Body wall	2·5	2·5	2·5
Salivary gland	0·06	0·08	0·1

* Quiescent.

labelled amino acid, and significant uptake was achieved within 5 min. The specific activities of proteins from these tissues were highest at 30 min and thereafter declined. Plasma proteins contained little radioactivity after 5 or 10 min, but reached maximum specific activity 90 min after injection.

In order to provide a basis for comparison of larvae differing in age, estimates of the total acid-insoluble protein of each tissue are presented for the stages studied (Table 1). The values for fat body, body wall, and salivary gland were based on purified homogenates of entire organs from two to three replicate groups of five animals. The determinations of total plasma protein involved similar purification of protein precipitated from measured aliquots of plasma; the total protein per larva was then calculated using the estimates of haemolymph volumes obtained by Kinnear *et al.* (1968). These values for the protein content of each tissue, together with the specific activities of protein at various times after injection of the radioisotope, provide a basis for quantitative comparison of the incorporation of [^{3}H]leucine into plasma proteins relative to other tissues (Fig. 2).

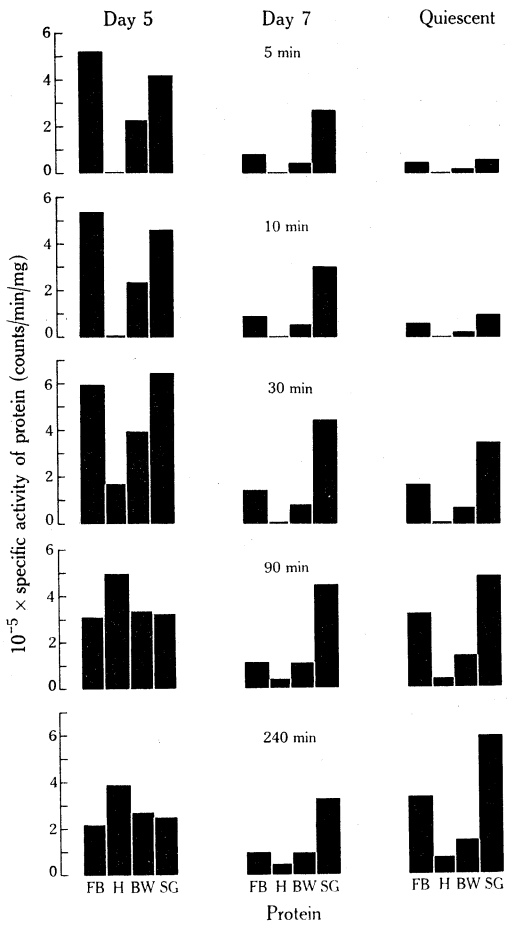

Fig. 1.—Time-course of incorporation of [^{3}H]leucine *in vivo* into various tissues of day-5, day-7, and day-11 (quiescent stage) larvae for specified intervals after injection. Each larva was injected with 2 μCi of [^{3}H]leucine, specific activity 23·5 Ci/m-mole, in 2 μl of distilled water. *FB*, fat body; *H*, plasma; *BW*, body wall; *SG*, salivary gland.

By comparison with the pattern of incorporation into protein in day-5 larvae, day-7 larvae showed much lower synthetic activity in fat body and body wall, and somewhat reduced uptake of radioisotope into salivary gland protein. The appearance of [^{3}H]leucine in fat body and plasma protein followed a similar pattern in day-5 and day-7 larvae, in that fat body protein reached peak specific activity at 30 min and thereafter declined, whilst the specific activity of plasma protein continued to increase. However, the specific activity of plasma protein did not exceed that of fat body protein in day-7 larvae after 30 min, as in day-5 larvae where such a relationship occurred at 90 min. Between days 5 and 7, the overall amount of plasma protein per larva increased fourfold. The total

radioactivity of the plasma protein therefore exceeded that of fat body in both day-5 and day-7 larvae at 90 and 240 min (Fig. 2).

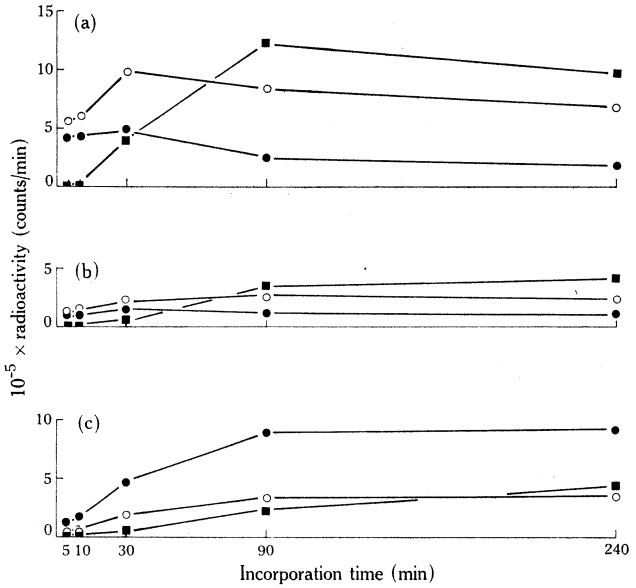

Fig. 2.—Comparison of total radioactivity per tissue accumulated at given times. (*a*) Day-5 larvae. (*b*) Day-7 larvae. (*c*) Day-11 larvae (quiescent stage).
● Fat body.
○ Body wall.
■ Plasma.

The specific activity of tissue proteins in quiescent larvae showed an initially slower increase compared with those of day-5 and day-7 larvae, but by 90 min after injection the specific activity of fat body protein had risen to a level significantly above that observed in day-7 larvae. Results of additional experiments with quiescent larvae over longer incorporation periods (240–480 min) are shown in Figure 3. The specific activity of fat body, body wall, and salivary gland protein increased up to 360 min after administration of the isotope, although plasma protein showed increased uptake of the radioisotope over the whole of this period.

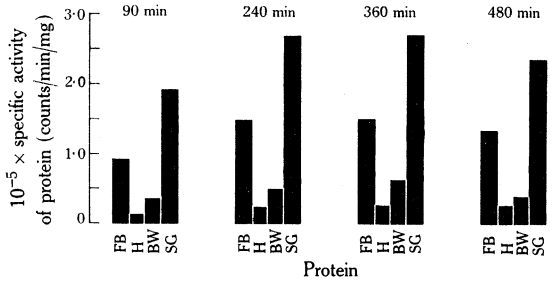

Fig. 3.—Time course of incorporation of [^{3}H]leucine *in vivo* into various tissues of day-11 larvae (quiescent stage) for specified intervals after injection. Each larva was injected with 1·5 μCi of [^{3}H]leucine, specific activity 14·7 Ci/m-mole, in 3 μl of distilled water. Abbreviations as in Figure 1.

IV. Discussion

Several interpretational problems arise in work on protein synthesis *in vivo*. These difficulties become aggravated in developmental studies by possible alterations in the balance between the factors involved, and because the various tissues may not

be affected in the same way or at the same time. For each tissue, the following points ultimately require analysis before a satisfactory understanding can be achieved:

(1) The relation of exogenous precursor to the endogenous amino acid pool accessible to the protein-synthesizing system in each tissue. Since our results revealed significant incorporation into those tissues studied, except haemolymph, after only a 5-min pulse (Fig. 1), ready mixing of labelled leucine in the endogenous pool of *C. stygia* is indicated. Although information is available on the free amino acid pools in *C. augur* (Hackman 1956) and in *Phormia regina* (Levenbook 1966), no comparable data is available for *C. stygia*. Levenbook and Dinamarca (1966) found up to a fivefold variation amongst individual larvae of *P. regina* in certain components of the endogenous amino acid pool. However, in the present experiments, consistent trends in total radioactivity of the various tissues were observed with increasing incorporation times, suggesting that gross patterns of protein synthesis were not obscured by individual variation of pool size under the conditions used.

(2) The dynamics of synthesis, retention, release (Price 1966), and uptake (Laufer and Nakase 1965) of protein in each tissue. There is also the possibility of differential uptake of amino acids by at least certain tissues (Price 1967).

(3) The relation between synthesis and breakdown or conversion, i.e. turnover rate.

(4) The magnitude and time-course of transitory stimulation of protein synthesis as a result of local injury, a factor known to have an effect in certain tissues of *C. stygia* (Neufeld, Thomson, and Horn 1968). The contribution of the injury effect to overall patterns of protein synthesis is difficult to assess in work where injection of an isotopic precursor must be followed by long incorporation periods, and where a differential response to injury might occur according to larval age.

Within the limitations imposed by lack of evidence on many aspects of these problems, the gross patterns of amino acid incorporation *in vivo* at different developmental stages should, nevertheless, reveal the basic features of synthetic activity in each tissue and elucidate certain significant interrelationships amongst the different tissues.

The principal developmental features and their relation to the observed patterns of protein synthesis in each larval tissue are as follows.

(i) *Fat Body*

This tissue is a major site of protein synthesis early in third instar, when protein reserves accumulate in the cells, which enlarge but do not divide. Late in the wandering stage, lipid globules appear in the cytoplasm. These functional changes can be correlated with marked alterations in the appearance of the polytene nuclei of the fat body cells (Thomson 1969). Similar increases in lipid and protein deposits occur in the fat body cells of *Drosophila melanogaster*, and in addition the

amount of glycogen per cell has been shown to rise during third instar (Butterworth, Bodenstein, and King 1965). The fat body in *C. stygia* comprises about 15% of the wet weight of the larva at day 5, 25% at day 8, and a little less at the quiescent stage (Martin and Kinnear, unpublished data). From day 5 to 11, the total acid-precipitable protein of this tissue increases approximately threefold (Table 1). In quiescent larvae, fat body protein amounts to 2·8 mg/larva, rising to 3·7 mg in the white prepupa. Difficulty is encountered with the latter determination as the fat body has commenced to break up at the prepupal stage.

It may be seen (Fig. 1) that the rate of uptake of radioactive amino acid into larval fat body protein is highest in day-5 larvae; incorporation is slower in day-7 and quiescent larvae. In view of the high synthetic activity of the fat body in day-5 larvae, the relatively small increase in precipitable protein from this stage to day-7 larvae is in agreement with the idea that much of the protein produced by the fat body during this period is not retained, but is released into the haemolymph. Although some synthetic activity takes place in quiescent larvae, accounting for at least part of the increase in fat body protein at this stage, transfer of protein from the haemolymph may also contribute. Uptake of plasma protein has been demonstrated in Lepidoptera (Loughton and West 1965; Locke and Collins 1968), and in Diptera (Laufer and Nakase 1965). In *C. stygia*, the observed drop in both the concentration and in the total amount of haemolymph protein at the end of larval life (Kinnear *et al.* 1968) is consistent with the occurrence of such uptake.

Price's (1966) *in vitro* studies on the incubation of the larval fat body of *C. erythrocephala* with [^{14}C]valine revealed that fat body from third-instar larvae released labelled protein into the incubation medium after an initial lag period. The released protein attained a higher specific activity than gross protein from the fat body itself. Price found a decrease in incorporation *in vitro* with increasing larval age throughout third instar. His data give no indication of an increase in protein synthesis in the fat body close to the time of puparium formation. Our data show that at incorporation times beyond 30 min, the specific activity of protein from all tissues of quiescent larvae is above that of day-7 animals. Neufeld, Thomson, and Horn (1968) showed that protein synthesis in the fat body and body wall of *C. stygia* larvae was influenced by exogenous moulting hormones at certain ages, so that rising endogenous levels of moulting hormone may be involved in the regulation of gross protein synthesis close to pupation.

(ii) *Haemolymph*

In early third instar (days 4–6), total plasma protein, carbohydrate, and lipid levels increase. From mid-third instar to just prior to the quiescent stage (i.e. from day 7 to 10) the total protein level declines, carbohydrate remains fairly constant, and lipid, which is at first constant, rises abruptly in the last 24 hr (Kinnear *et al.* 1968). In quiescent larvae, the total plasma protein declines still further, carbohydrate increases, and lipid decreases. It appears that the main site of haemolymph protein synthesis is the fat body. This has been demonstrated in the silkworm *Bombyx mori* by Shigematsu (1958), in the giant silk moths *Hyalophora cecropia* and *Samia cynthia* by Laufer (1960), and in the blue blowfly *C. erythrocephala* (Price 1966; Price and Bosman 1966). The fat body and haemolymph proteins of *C. stygia* are similar in

electrophoretic mobility (Kinnear, Martin, and Thomson, unpublished data). *In vitro* studies on larval fat body from *C. erythrocephala* have shown the protein released into the incubation medium to be electrophoretically similar to haemolymph protein (Price and Bosman 1966).

The high synthetic activity of fat body at day 5 compared with later ages may be correlated with a rapid increase in plasma protein concentration: from approximately 70 mg/ml at day 5 to approximately 160 mg/ml at day 6, concurrent with an increase in haemolymph volume. This is also the period of maximum growth of the larva, when the weight increases from about 65 mg/larva at day 5 to 115 mg/larva at day 6 (Kinnear *et al.* 1968).

The labelling pattern of plasma differs from that of the other tissues in that there is a delay in the incorporation of the isotope into the plasma proteins at each age examined. Further, the specific activity of plasma protein continues to rise after that of the protein from the fat body begins to fall (see Figs. 1 and 3). It is thus apparent that protein is released into the haemolymph. A similar lag in the labelling of haemolymph proteins was found by Skinner (1963) with the giant silkworm *H. cecropia*, whilst Faulkner and Bheemeswar (1960) showed in a study of *B. mori* that the rate of incorporation of [^{14}C]glycine into blood proteins, unlike that of other tissues, exhibited a delay before becoming linear.

If it is assumed on these grounds that the fat body is the major site of synthesis of plasma protein during the third instar in *C. stygia*, it is clear that the amount of newly synthesized protein retained, relative to that released by the fat body, changes markedly between days 7 and 11. In both day-5 and day-7 larvae, total radioactivity of plasma protein exceeds that of fat body protein 90 and 240 min after administration of the isotope (Fig. 2). In day-11 larvae, the total radioactivity of plasma protein remains below that of fat body protein even to 480 min after injection (Fig. 3 and Table 1).

The decline in specific activity of plasma protein between the 90- and 240-min incorporation times in day-5 larvae reflects the sharp increase in plasma protein at this stage as well as rapid utilization of the injected radioactive leucine. During this interval a drop in the estimated total number of counts present in plasma protein appears to take place, suggesting that significant turnover of at least some plasma proteins may occur.

In the present work, no attempt has been made to investigate changes of the cellular components of haemolymph.

(iii) *Body Wall*

The protein content of the body wall remains approximately constant subsequent to day 5 (Table 1), indicating that there is neither marked retention of newly synthesized protein nor massive uptake in this tissue. The rate and amount of incorporation into body wall protein drops markedly in day-7 and quiescent larvae, compared with day-5 larvae. The abrupt and profound changes in consistency and then colour of the cuticle, which terminate the quiescent stage and mark the onset of puparium formation, are thus not preceded by a gross change in the amount of protein synthesis in the epidermis and associated tissues. Even so, at least one

specific enzyme involved in sclerotization appears to be induced in epidermal cells by the rise in titre of moulting hormone which occurs at this stage (Sekeris 1965).

(iv) *Salivary Glands*

These are relatively large organs in *Calliphora*, their size being probably related to considerable extracorporeal digestion of the food during the active feeding stage. In the post-feeding period of the third instar, the glands continue to produce a secretion, but cytoplasmic accumulation of protein becomes more marked, and the protein content of the salivary tissue increases gradually throughout third instar (Table 1). In comparison with the other tissues examined, the specific activities of salivary gland proteins from both day-7 and quiescent larvae attain maximum values fairly close to that observed in day-5 larvae, but do so more slowly, especially at the quiescent stage. These changes in the incorporation pattern of the salivary gland suggest a gradual reduction in the rate of uptake of radioactive amino acid during third instar, coupled with increasing retention of newly synthesized protein. Studies on *Chironomus* have shown that salivary gland secretory products include proteins which have been transported to the cells from the haemolymph, as well as proteins produced *in situ* (Laufer 1968). In the present work, the specific activities of salivary gland proteins follow the same trend as in fat body and body wall tissues: no delay occurs in the labelling of these proteins, and hence there is no evidence for large scale transport of protein to the salivary gland in *C. stygia*.

Our experimental results show that the rate of synthesis of protein in fat body, body wall, and salivary gland tissues over the first 30 min is much greater in day-5 than in either day-7 or quiescent larvae. A similar decrease in synthetic activity with age in *C. erythrocephala* was postulated by Price and Munn (quoted by Price 1968) to be reflected by a decrease during third instar in the ribosomes of the endoplasmic reticulum. Sekeri, Sekeris, and Karlson (1968) found a decrease in the proportion of ribosomal particles in the form of heavy polysomes when 6–7-day (post-feeding) larvae of *C. erythrocephala* were compared with 8-day larvae and white prepupae.

In general, protein synthesis is greater in each tissue studied in day-5 than in day-7 or quiescent larvae. The rate of uptake of [^{3}H]leucine into fat body protein is minimal in day-7 larvae and rises to an intermediate level at day 11. The synthesis in fat body of the proteins which are apparently liberated into haemolymph is largely shut off after day 5. In quiescent larvae, the incorporation pattern of fat body protein bears a different relationship to that of haemolymph; the fat body increasingly retains newly synthesized proteins. Two major changes in the biosynthetic functions of fat body are therefore suggested by the pattern of protein synthesis in this tissue, one occurring between days 5 and 7, the other between day 7 and the quiescent stage. Both presumably reflect coordinated switching of the genetic units involved. Protein synthesis in the tissues of the body wall is markedly reduced after day 5, and increases only slightly at the quiescent stage, so that no abrupt change in the overall pattern of gene activity in this tissue during later larval life is indicated. Unlike fat body and body wall, the salivary gland maintains a relatively high level of protein synthesis throughout third instar. However, the incorporation pattern suggests more rapid amino acid uptake and quick release of protein at day 5, with

slower synthesis and increased retention at the quiescent stage. The significance of these apparent changes in salivary gland will, however, only become apparent when qualitative studies of the salivary gland proteins of *Calliphora* have been completed.

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