PROTEIN METABOLISM BY THE SALIVARY GLANDS AND OTHER ORGANS OF THE LARVA OF THE BLOWFLY, CALLIPHORA ERYTHROCEPHALA

GARETH M. PRICE

Unit of Invertebrate Chemistry and Physiology, Agricultural Research Council, University of Sussex, Brighton BN1 9QJ, Sussex, England

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Abstract—Protein metabolism in salivary glands, gut, haemolymph, and fat body during the last larval instar of the blowfly, Calliphora erythrocephala, has been investigated. In salivary glands, protein release, protein synthesis, amylase, and pepsin-like protease activity were maximal in 6 day larvae, this being at a time when the larvae had finished feeding. All these functions declined in glands from the rounded-off white puparial stage (R.O.) while acid phosphatase activity rose throughout the third instar to a maximum at the R.O. stage. Glands from 6 and 7 day larvae released protein which on disk gel electrophoresis separated into four minor bands and two major bands one of the latter possessing protease activity.

In the gut, pepsin-like protease activity was maximal in 4 day larvae after which it fell rapidly thus following the feeding pattern of the larva in contrast to that in the salivary glands which did not.

In vitro experiments showed that protease was released from 6 day glands through the basal membrane of the cells and not via the duct. A pepsin-like protease was also found in the haemolymph and fat body, the activity in the fat body rising rapidly during the latter part of the third instar, a rise which is attributed to the fat body sequestering protease from the haemolymph. Acid phosphatase activity in the fat body was maximal in 5 day larvae indicating that this enzyme was synthesized early in the third instar. It was shown that fat body sequestered 14C-labelled protein synthesized by and released from the salivary glands, most of the 14C activity being associated with a 600 g precipitable, acid-phosphatase rich fraction.

It is proposed that in late third instar larvae the salivary glands function as glands of internal secretion, releasing protease into the haemolymph, which is then sequestered by the fat body (and perhaps other tissues) and is subsequently used in the lysis of the tissues at the time of metamorphosis.

INTRODUCTION

There is now abundant evidence that in insects as in other animals the salivary glands play a predominantly digestive role. The saliva secreted by them is expelled from the mouth to moisten the food on which the insect is feeding and the enzymes it contains partially digest the food which is then taken into the mouth in a semi-fluid state. Numerous enzymes have been detected in saliva (WIGGLESWORTH,
1965); however, there is also evidence that the saliva of some insects does not contain any enzymes, digestion in these insects being carried out by enzymes originating in the gut. Examples are the blood-feeding Tabanid, *Chrysops silacea* (WIGGLESWORTH, 1931), the tsetse fly, *Glossina* (WIGGLESWORTH, 1929), and the blood-sucking bugs, *Cimex, Rhodnius*, and *Triatoma* (BAPTIST, 1941). In these insects the saliva contains an anticoagulant. In some insects little is known about the rôle played by their salivary glands, especially and surprisingly those of *Drosophila* (ASHBURNER, 1970; LANE et al., 1972).

An an early stage in the present work it became apparent that the salivary glands in third instar blowfly larvae exhibit maximum activity (protein synthesis, release of protein, enzyme activity) after the larvae have finished feeding. This observation indicated that in late larvae the salivary glands must be playing some rôle other than that in the digestion of food. This possibility has been examined and the protein metabolism of the gland is considered in relation to that of the gut, haemolymph, and fat body. A preliminary account of some of this work is in press (PRICE, 1973a).

**MATERIALS AND METHODS**

**Chemicals**

The following chemicals were purchased. Acrylamide and N,N'-methylenebis-acrylamide (MBA) of electrophoresis-purity grade from Bio Rad Laboratories, 32nd and Griffin, Richmond, Calif., U.S.A.; NNN’-tetramethyl-1,2-diaminoethane (TEMED) from B.D.H. Chemicals, Poole, Dorset; haemoglobin (Merck) from Anderman and Co. Ltd., East Moseley, Surrey; [U-14C] valine (sp. act. 6.9 mCi/mM) from the Radiochemical Centre, Amersham, Bucks.; 2,5-diphenyl-oxazole (PPO), 1,4-Di[2-(5-phenyloxazolyl)] benzene (POPOP) and human γ-globulin from Koch-Light Laboratories Ltd., Colnbrook, Bucks. All other chemicals were of Analar grade and glass-distilled water was used throughout.

**Breeding of Calliphora**

*Calliphora* larvae were bred as previously described (PRICE, 1969).

**Isolation of salivary glands**

Salivary glands and other tissues were isolated under a Ringer’s medium as previously described (PRICE, 1972).

**Extraction of salivary glands for assay of protein**

Ten glands were homogenized in 1 ml of cold 0.4 N perchloric acid in an all-glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 2700 g for 15 min, the supernatant discarded, and the residue dispersed in 1ml of 0.4 N HClO₄ and centrifuged as before. This residue was well drained and dispersed in 1 ml of 0.3 N KOH with which it was incubated for 1 hr at 37°C during which time nearly all of the residue usually dissolved. Any insoluble material remaining was
centrifuged down and the protein content of the supernatant was assayed by the method of Lowry et al. (1951). γ-Globulin was used as standard.

**Disk electrophoresis on polyacrylamide gel**

Disk electrophoresis was carried out in a conventional manner using stacking gels and separating gels having final acrylamide concentrations of 2.5 and 5%, respectively. The buffer in the upper (cathode) compartment was Tris 0.05 M, glycine 0.046 M, pH 8.9, and in the lower (anode) compartment was Tris 0.1 M, HCl 0.05 M, pH 8.1. Electrophoresis was carried out for 5 to 10 min at 3 mA/tube until the sample entered the separating gel and then continued for approximately 2 hr at 4 mA/tube, all operations being carried out in the cold. After electrophoresis the gels were immersed for 2 hr in a 7% solution of acetic acid containing amido black to 0.1% and coomassie blue to 0.02%. For destaining, the gels were transferred to a 7% solution of acetic acid and left for 24 hr with constant stirring of the solution.

**Protein synthesis by salivary glands**

Twenty glands were incubated for 1 hr at 30°C in a Ringer’s medium (Price, 1972) containing a mixture of amino acids and [U-14C]-valine (Price, 1969) all at pH 7.2. At the end of the incubation period the glands were removed from the medium and homogenized in 2 ml of 0.4 N HClO4. Further extraction procedure and estimation of protein was carried out as described above.

**Determination of 14C activity**

A sample (100 μl) of the protein-containing KOH extract was added to 10 ml of scintillation fluid (PPO 4 g; POPOP 0.1 g; toluene 700 ml; 2-ethoxyethanol 300 ml) and its activity was measured over a 20 min period in a Packard Tricarb scintillation counter. All counts were corrected for background activity.

**Assay of enzyme activity**

(1) **Amylase activity**

(a) In Calliphora salivary glands: Ten glands were homogenized in 1 ml of Ringer’s medium in an all-glass Potter-Elvehjem homogenizer and the homogenate was frozen by dipping the tube into an acetone-solid CO2 mixture. It was thawed under warm water, the process of freezing and thawing being carried out five times. The frozen and thawed homogenate was centrifuged at 2700 g for 15 min and amylase activity in the supernatant was assayed by the method of Bernfeld (1955). In this assay, and in the assays of other enzymes described below, the absorption of zero time control samples was always deducted from that obtained with the incubated samples and absorption was measured against a blank containing Ringer’s medium in place of the enzyme extract. All determinations were carried out at least in duplicate. Amylase activity is expressed as mg of maltose produced per mg of protein or per gland per hr. In this paper the term 'per gland' refers to the pair of glands obtained from a single larva.
(b) In incubation medium: Salivary glands from larvae of different ages were incubated in groups of 10 in 1 ml of Ringer's medium for 3 hr at 30°C. They were removed and the amylase activity in the medium was assayed as described under (a).

(c) In *Calliphora* plasma: A small cut was made in the anterior end of the larva and the haemolymph released (about 10 μl) was collected in an ice-cold tube. About 0.5 ml was centrifuged at 2700 g for 15 min to precipitate the blood cells. Amylase activity in a sample of the plasma (50 μl) was assayed as described under (a).

(d) In cockroach salivary glands: Salivary glands were isolated from three final instar female larvae of the cockroach, *Periplaneta americana*, and homogenized in 1 ml of Ringer's medium. The homogenate was centrifuged at 2700 g for 15 min and amylase activity in a sample of the supernatant was assayed as described under (a).

(e) In human saliva: A 50 μl sample of saliva was diluted to 2.0 ml with Ringer's medium and the amylase activity in 50 μl of this solution was assayed as described under (a).

(2) Acid phosphatase activity

(a) In *Calliphora* salivary glands: Ten glands were homogenized in 1 ml of Ringer's medium, the homogenate frozen and thawed five times and centrifuged as described above. The acid phosphatase activity in samples (0.2 ml) of the supernatant was assayed as described by Henrikson and Clever (1972). Acid phosphatase activity is expressed as the change in o.d. at 410 nm per gland per hr.

(b) In *Calliphora* fat body: Two fat bodies were homogenized in 1 ml of Ringer's medium, the homogenate frozen and thawed and centrifuged. The acid phosphatase activity in 0.2 ml samples of the supernatant was assayed as described under (a).

(3) Protease activity

(a) In *Calliphora* salivary glands: Twenty glands were homogenized in 1 ml of Ringer's medium, the homogenate frozen and thawed five times, and centrifuged. Protease activity in samples (0.1 ml) of the supernatant was assayed as described by Henrikson and Clever (1972). Protease activity is expressed as the change in O.D. at 700 nm per gland per hr.

(b) In incubation medium: Ten glands were incubated in 0.5 ml of Ringer's medium for 3 hr at 30°C. They were removed and the protease activity in 0.1 ml samples of the medium was assayed as described under (a).

(c) In plasma: Plasma was isolated as described above and the protease activity in 50 μl samples was assayed as described under (a).

(d) In midgut: Tissues were isolated as previously described (Price, 1972). The midguts from 10 larvae were homogenized in 1 ml of Ringer's medium, frozen and thawed, centrifuged, and protease activity in 0.1 ml samples of the supernatant was assayed as described under (a).

(e) In fat body: Fat bodies, isolated from two larvae, were homogenized in 1 ml
of Ringer's medium, frozen and thawed, centrifuged, and protease activity in 0.1 ml samples of the supernatant was assayed as described under (a).

RESULTS

Microscopic appearance of Calliphora salivary glands

The main features of the paired salivary glands and associated structures from a 7 day Calliphora larva are shown in Fig. 1, which also shows the gland arranged for an experiment described later in the paper. The gland is comprised of two sack-like arms (a1 a2) each arm being a tube 6 to 7 mm long and 0.8 mm in diameter.

The walls of each tube are made up of a single layer of large cells, 150 to 200 µm in diameter, each with a large nucleus, 70 to 80 µm in diameter. The lumen of the tube ends blindly at the posterior end while at the anterior end it opens into a duct, 0.5 to 0.6 mm long, which joins with the duct from the other arm to form a common salivary duct (d). Midway along their length the arms are bridged by a garland of binucleate cells (g) termed nephrocytes (KEILIN, 1917; WIGGLESWORTH, 1965; CROSSLEY, 1972), while at their posterior borders the arms are joined by a bridge of fat body cells (fb). Unless otherwise stated, the fat body cells were carefully removed when the glands were isolated and thus were not included in any of the assays.

Protein content of salivary glands

Isolation of glands and assay of protein was carried out as described in the Materials and Methods section. The protein content (Fig. 2) rose from 42 µg/gland in 4 day larvae to a maximum of 90 µg in 6 day larvae and thereafter fell to
55 μg at the rounded-off white puparial stage (R.O.). Under the culturing conditions employed the R.O. stage occurred at approximately 8 days. Measurements of the protein content at stages later than R.O. were not reliable because the glands tended to disintegrate during their isolation.

![Graph showing protein content vs age](image)

**Fig. 2.** Protein content of salivary glands from *Calliphora* larvae of various ages. The term 'per gland' refers to the pair of glands from one insect. In all figures where applicable, R.O. is the rounded-off white puparial stage. Details of extraction of the gland and assay of protein are given in the text.

**Secretion of protein by salivary glands**

Glands were isolated from larvae of various ages and groups of 10 were incubated with gentle shaking in 1 ml of Ringer's medium at 30°C for 3 hr. At the end of the incubation period the glands were removed and the protein content of the medium assayed. The amount of protein released by the glands increased as the larvae aged (Fig. 3) reaching a maximum in 6 to 7 day larvae, the amount released then falling to a low level at the R.O. stage. It was found that glands isolated from 7 day larvae released protein at a linear rate over a period of 5 hr.

**Electrophoresis of proteins**

(a) In incubation medium: Groups of 10 glands from larvae of different ages were incubated in 0.5 ml of Ringer's medium for 3 hr at 30°C. They were removed and 200 μl of the medium was subjected to electrophoresis as described in the Materials and Methods section. Three major bands of protein were clearly discernible on gels from 4 and 5 day larvae (Fig. 4a) and in 6 and 7 day larvae the two slower running of these bands became more intense while the faster of the three bands decreased in intensity. Later, at the R.O. stage, all three bands were hardly discernible.
(b) In a homogenate of salivary glands: Groups of 10 glands were homogenized in 0.5 ml of Ringer's medium. The homogenate was centrifuged at 2700 g for 15 min and 200 μl samples of the supernatant were subjected to electrophoresis. The gels of the gland extract (Fig. 4b) contained proteins whose electrophoretic behaviour was similar to that of proteins released into the incubation medium (Fig. 4a), and they also contained a number of slower running bands which were not discernible in the incubation medium. As with the incubation medium there was a striking reduction in the intensity of the major bands at the R.O. stage.

![Graph showing protein secretion by salivary glands from Calliphora larvae of various ages.](#)

Fig. 3. Protein secretion by salivary glands from Calliphora larvae of various ages. Incubation conditions are given in the text.

![Polyacrylamide-gel electrophoretic patterns of proteins.](#)

Fig. 4. Polyacrylamide-gel electrophoretic patterns of proteins: (a) released by salivary glands and (b) in homogenates of glands from Calliphora larvae of various ages. O, Origin of separating gel; md, marker dye. Arrow shows the direction of migration. The amido black staining intensity of each band is illustrated by the density of shading. Details of incubation and electrophoresis are given in the text.
Protein synthesis

Protein synthesis was maximal in glands from 6 day larvae, the rate subsequently falling over the remainder of the larval period (Fig. 5). After incubation for 1 hr, 90 per cent of the total radioactivity incorporated into protein was present in the gland and 10 per cent was present in protein released into the medium. Fig. 5 shows the specific radioactivity of protein in the gland. The curve of protein synthesis (Fig. 5) is similar to that of protein content (Fig. 2), both parameters being maximal in glands from 6 day larvae.

Amylase activity

(a) In Calliphora salivary glands: Amylase activity increased as the larvae aged from 4 to 5 days, reached a maximum in 6 day larvae, and fell in 7 day larvae finally reaching a minimum level at the R.O. stage (Fig. 6).

![Graph showing protein synthesis](image1)

**Fig. 5.** Incorporation of [U-14C] valine into protein by salivary glands from Calliphora larvae of various ages. Details of incubation and assay of protein and radioactivity are given in the text.

![Graph showing amylase activity](image2)

**Fig. 6.** Amylase activity in salivary glands from Calliphora larvae of various ages. O—O, Activity in homogenate of gland; △—△, activity in incubation medium. Details of incubation and enzyme assay are given in the text.
(b) In incubation medium: The level of amylase activity found in the medium after glands from larvae of different ages were incubated for 3 hr at 30°C is also shown in Fig. 6. The curve is similar to that obtained for the gland extract, both curves attaining a maximum in 6 day larvae and falling to a low level at the R.O. stage.

(c) In *Calliphora* plasma: Amylase activity in *Calliphora* plasma increased during the third larval instar, reached a maximum level in 7 day larvae, and remained at this level at the R.O. stage (Fig. 7). The total protein content of the plasma over the same period is also shown in Fig. 7. The protein concentration was highest (115 mg/ml) in 5 and 6 day larvae subsequently falling to 78 mg/ml at the R.O. stage.

(d) In *Sarcophaga* and *Periplaneta* salivary glands and human saliva: Glands were isolated from larvae of *S. barbata* in an identical way to that described for *Calliphora*. The levels of activity are shown in Table 1. Of the insect salivary glands those of the cockroach possessed strong amylase activity whereas those of *Calliphora* and *Sarcophaga* possessed very weak activity. The activity in their plasma was also weak. The highest activity was found in human saliva, it being seven times as active as the gland extract of the cockroach.

**Acid phosphatase activity**

(a) In *Calliphora* salivary glands: Acid phosphatase activity increased throughout the third larval instar reaching a maximum at the R.O. stage (Fig. 8). Assays were not attempted at stages later than the R.O. stage because the salivary glands tended to disintegrate during their isolation.
TABLE 1—Amylase activity in salivary glands and in plasma from last larval instars of some insects and in human saliva

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue</th>
<th>Amylase activity (mg of maltose produced/mg of protein per hr at 30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calliphora erythrocephala</td>
<td>Salivary glands</td>
<td>0.33</td>
</tr>
<tr>
<td>Calliphora erythrocephala</td>
<td>Exudate of glands</td>
<td>2.00</td>
</tr>
<tr>
<td>Calliphora erythrocephala</td>
<td>Plasma</td>
<td>1.00</td>
</tr>
<tr>
<td>Sarcophaga barbata</td>
<td>Salivary glands</td>
<td>0.23</td>
</tr>
<tr>
<td>Sarcophaga barbata</td>
<td>Plasma</td>
<td>0.81</td>
</tr>
<tr>
<td>Periplaneta americana</td>
<td>Salivary glands</td>
<td>2418.0</td>
</tr>
<tr>
<td>Human</td>
<td>Saliva</td>
<td>17300.0</td>
</tr>
</tbody>
</table>

Fig. 8. Acid phosphatase activity in salivary glands from Calliphora larvae of various ages. Details of the enzyme assay are given in the text.

When glands from 7 day larvae were incubated for 3 hr at 30°C in Ringer's medium no acid phosphatase was detectable in the medium.

(b) In Calliphora fat body: Acid phosphatase activity was maximal in fat body from 5 day larvae thereafter falling slightly over the remainder of the instar (Fig. 9). When homogenates of fat body from 7 day larvae were frozen and thawed two, four, six, eight, and ten times the amount of activity recovered in the supernatant was the same in each case. Since the results in Fig. 9 were obtained with preparations frozen and thawed five times then it is unlikely that the slight fall in activity as the larvae age is due to less efficient recovery from older fat body, but is a true reflection of the level of activity.

Protease activity

(a) In Calliphora salivary glands: It was found that protease activity was near maximal at pH 2.5 to 3.0, the activity falling rapidly above pH 3.0 to virtually zero
Fig. 9. Acid phosphatase activity in fat body from Calliphora larvae of various ages. Details of isolation of the fat body and of the enzyme assay are given in the text.

Fig. 10. Effect of pH on protease activity of salivary glands from 7 day Calliphora larvae. Details of incubation and of enzyme assay are given in the text.

at pH 6-5 (Fig. 10). This result indicated the presence of a pepsin-like protease and subsequent assays were carried out at pH 3-0. The levels of protease activity in glands from larvae of different ages and in Ringer's medium in which glands were incubated for 3 hr are shown in Fig. 11. Protease activity in the gland increased as the larvae aged from 4 to 6 days and was at a maximum in glands from 6 and 7 day larvae, subsequently falling to a low level at the R.O. stage. Glands from 6 and 7 day larvae released as much protease in 3 hr as was present in freshly isolated glands. After electrophoresis of samples of the medium it was found that protease activity
in the gel coincided with the slower running of the two major protein bands revealed on parallel gels by staining with amido black (see Fig. 4a). In a similar experiment in which [U-14C] valine was included in the incubation medium, most of the 14C activity in the gel coincided with the two major protein bands.

(b) In midgut: Over the period investigated, pepsin-like protease activity (assay at pH 3.0) was highest in 4 day larvae, fell rapidly in 5 and 6 day larvae, and reached its lowest level in 7 day larvae and rounded-off white puparia (Fig. 12). Thus the level of protease activity in the midgut reflects the feeding pattern of the larva. Under the culturing conditions employed the larvae feed voraciously until
they are 4 to 5 days old. On the fifth day they leave the meat on which they have been feeding and by the sixth day their crops are empty. Though the level of protease activity in the midgut reflects the feeding pattern of the larva, that in the salivary glands does not, for the level of protease released by the glands and that in the gland extract are maximal in 6 to 7 day larvae (Fig. 11), that is, at a time when the larvae have stopped feeding. This result suggested that the protease released by the salivary glands was not playing any rôle in digestion of food.

(c) Pathway of release of protease: If the protease released by the salivary glands was not playing any rôle in digestion then it seemed possible that it was being secreted not via the duct of the gland but directly through the basal membrane of the gland cells. To test this possibility glands were set up as shown in Fig. 1. Glands were isolated very carefully to avoid injuring them and immersed in a globule (50 μl) of Ringer's medium under liquid paraffin. The duct was pulled free of the globule so that its open end projected into a second globule. The pins prevented the two globules from coalescing. Several glands were incubated in this manner at room temperature for 3 hr after which samples (25 μl) of the medium were taken from the gland end and from the duct end and assayed for protease activity. Activity was found only in samples from the gland end.

(d) In plasma: It was found that the plasma contained a pepsin-like protease, its pH activity curve being identical to that of the salivary gland protease (Fig. 10). Assay at pH 3.0 showed that protease activity in the plasma increased as the larvae aged from 4 to 5 days after which it steadily declined over the remainder of the larval period (Fig. 13). Since the pH of blowfly larval haemolymph is 7.4 and since at this pH protease activity was not detectable, then it is unlikely that the protease is active while in the haemolymph.

(e) In fat body: Like the salivary glands and haemolymph, fat body contained a pepsin-like protease, the level of which increased as the larvae aged and, over the period measured, reached a maximum level at the R.O. stage (Fig. 14).

![Graph](image-url)  
**Fig. 13.** Protease activity in plasma from *Calliphora* larvae of various ages. Details of isolation of the plasma and of enzyme assay are given in the text.
Uptake of $^{14}$C-labelled protein by fat body

Twenty salivary glands from 6 day Calliphora larvae were incubated in 1.0 ml of plasma containing 1 $\mu$Ci of [U-$^{14}$C] valine and a few crystals of phenylthiourea for 18 hr at 25°C. The glands were removed and the plasma was passed down a column (10 x 1 cm) of Sephadex G-25 which had previously been equilibrated with Ringer's medium. The protein fraction (1.7 ml) was collected and in it 10 fat bodies from 7 day larvae were incubated for 4 hr at 25°C. The fat bodies were removed, well washed in a large volume of Ringer's medium, homogenized in 5.0 ml of fresh medium, and the homogenate fractionated by differential centrifugation as shown:

Homogenate of 10 fat bodies (i):
Centrifuged at 600 g for 15 min

- 600 g supernatant (ii)
  Centrifuged at 2000 g for 15 min
  - 2000 g supernatant (v)
    Centrifuged at 10,000 g for 15 min
      - 10,000 g supernatant (vii)
  - 600 g residue
  Centrifuged at 2000 g for 15 min
    - 2000 g residue (vi)
      Resuspended in 1.0 ml of Ringer's medium
      - 10,000 g residue (viii)
        Resuspended in 1 ml of Ringer's medium
    - 600 g washings (iv)
      Resuspended in 600 g centrifuged at 10,000 g for 1.5 min
        - 10,000 g supernatant
          Resuspended in 1.0 ml of Ringer's medium

SCHEME 1. Flow diagram showing treatment of fat body homogenate. The numbers in parentheses relate to the enzyme activity and $^{14}$C activity shown in Fig. 15.
in Scheme 1. Fig. 15 shows that the distribution of acid phosphatase activity and $^{14}$C activity between the various fractions was similar with a major part of the enzyme activity and the radioactivity being associated with a 600 g precipitable fraction.

![Graph showing acid phosphatase activity and $^{14}$C activity](image)

**Fraction no. (see scheme 1)**

Fig. 15. Acid phosphatase activity (a) and $^{14}$C activity (b) in various fractions obtained from a homogenate of fat body from 7 day *Calliphora* larvae. (The numerals on the abscissa refer to the fractions obtained as shown in Scheme 1. Details of incubation of the fat body, and of assay of enzyme activity and of radioactivity are given in the text.

**DISCUSSION**

The amount of protein found in salivary glands from 6 day *C. erythrocephala* larvae (90 µg, Fig. 2) agrees well with that found (80 µg) in glands from 7 day larvae of *C. stygia* by MARTIN *et al.* (1969). However, these authors found that as the larvae aged from 7 to 11 days the protein content of the glands increased from 80 to 100 µg whereas in the present work with *C. erythrocephala* the protein content decreased from 90 µg in 6 day larvae to 55 µg at the rounded-off white puparial stage (R.O., Fig. 2). Changes in protein content have also been observed in *C. tentans*, the level rising from 4 to 20 µg/gland during the last larval instar (DARROW and CLEVER, 1970) and in *C. thummi* where the level rises from 4-65 µg/gland in mid-fourth instar larvae to 12-4 µg in early pupae and then falls to 8-1 µg at a later stage (LAUFER, 1968). The amount of protein released by *C. erythrocephala* glands was maximal in 6 and 7 day larvae (Fig. 3), that is, at a time when the larvae had stopped feeding and the rate of release *in vitro* was linear for up to 5 hr.

After disk electrophoresis of salivary gland extracts from fourth instar larvae of *C. tentans*, DOYLE and LAUFER (1969) found 11 major protein fractions, 10 of which
were also found in the haemolymph. Working with *C. stygia*, Kinneir *et al.* (1971) found that electrophoresis of a salivary secretion from glands of third instar larvae separated the protein into 18 bands some of which were present at the feeding stage only to disappear at the wandering stage and vice versa. In the present work with *C. erythrocephala*, three major bands were found at the feeding stage (Fig. 4a; 4, 5) and some minor bands were just discernible, while at the wandering stage (Fig. 4a; 6, 7) the fastest running of the three major bands decreased in intensity and the two slower running bands increased. At the R.O. stage the major bands were virtually absent. Electrophoresis of a Ringer's extract of the gland revealed the major bands seen in the secretion and some slow-running bands (Fig. 4b; 6, 7) not discernible in the secretion. Again at the R.O. stage there was a loss in the major bands. The absence of the major bands from the secretion and from the gland at the R.O. stage indicates that at this stage the gland has stopped synthesizing and secreting these proteins.

The rate of protein synthesis was maximal in glands from 6 day larvae, the rates in 5 and 7 day larvae being only slightly less (Fig. 5). This result is in contrast to that obtained with fat body (Price, 1966) where the rate falls rapidly as the larvae age from 4 to 5 days and reaches a low level in 6 and 7 day larvae. Similar results were obtained by Martin *et al.* (1969) with *C. stygia*.

The level of amylase activity in salivary glands and the amount released by them was greatest in 6 and 7 day larvae (Fig. 6). Thus, as with protease the highest level of activity was attained after the larvae had finished feeding indicating that the amylase released by the glands of the late third instar larvae might not be playing any role in the digestion of food. The highest levels of activity found in glands from Calliphora and Sarcophaga larvae were still very low compared with those found in Periplaneta glands and human saliva (Table 1). In the plasma the level of amylase activity rose throughout the third instar (Fig. 7) and did not fall at the R.O. stage as it did in the salivary glands. The level of total plasma protein fell from 155 mg/ml in 6 day larvae to 78 mg/ml at the R.O. stage, a fall which is probably due to the sequestration of protein by the fat body over this period (Kinneir *et al.*, 1968; Martin *et al.*, 1971).

Acid phosphatase activity in salivary glands increased throughout the third instar reaching its highest level at the R.O. stage (Fig. 8). An increase in acid phosphatase activity has also been observed in glands during the last larval instar and early pupal period of *C. tentans* (Laufer and Schin, 1971). When glands from 7 day Calliphora larvae were incubated for 3 hr in a Ringer's medium no acid phosphatase was detectable in the medium indicating that the enzyme was not released by the glands. This was not an unexpected result for it has been shown that in salivary glands from other dipterous larvae, acid phosphatase is located in intracellular structures closely resembling vertebrate lysosomes (Schin and Clever, 1965).

In the fat body, acid phosphatase activity was highest in 5 day larvae, subsequently decreasing as the larvae aged (Fig. 9). This suggests that most, if not all, of the acid phosphatase is synthesized during the early period of the third instar, this
also being the period of maximum overall protein synthesis by the fat body (PRICE, 1966). With fat body from 7 day larvae much of the activity was associated with a 600 g precipitable fraction (Fig. 15) which was found to be rich in large (1 μm) granules. Such granules have previously been observed in fat body from larvae of this age (PRICE, 1969) and as there is evidence that they are lysosomes (BENSON, 1965) it is reasonable to assume that in the 600 g fraction they contain the acid phosphatase. Although large granules are absent from fat body of younger larvae (PRICE, 1969) it possesses a high level of acid phosphatase activity (Fig. 9). This suggests that in young larvae acid phosphatase is present either in much smaller granules which later increase in size, or it is located 'free' in the cytoplasm to be subsequently taken up by granules formed later in the instar. This aspect is currently under investigation.

Pepsin-like protease activity was maximal in glands from 6 and 7 day larvae (Fig. 11) whereas in the midgut it was maximal in 4 day larvae (Fig. 12), thereafter falling to a low level in 7 day larvae. Pepsin-like proteases have previously been demonstrated in gut extracts of *Musca domestica* (GREENBERG and PARETSKY, 1955), *Stomoxys calcitrans* L. (LAMBERT et al., 1959), and *Calliphora vomitoria* L. (FRASER et al., 1961). The activity in the gut followed the feeding pattern of the larvae but that in the salivary glands did not. This suggested that the protease released by the glands from late third instar larvae was not playing any rôle in the digestion of food, a possibility that received support when it was found that, *in vitro*, protease was released not via the duct but directly through the basal membrane of the cells of the gland. If this *in vitro* result reflected the *in vivo* situation then it meant that *in vivo* the salivary gland secretes protease into the surrounding haemolymph, that is, it would be functioning as a gland of internal secretion. Movement of protein in the opposite direction, that is, from the haemolymph into the salivary glands, has been shown to take place in larvae of *C. thummi* (LAUFER and NAKASE, 1965; LAUFER, 1968) and *C. tentans* (DOYLE and LAUFER, 1969). Working with *C. tentans*, RODEMS et al. (1969) found that in salivary glands from last instar larvae, protease activity was maximal at pH 5.5 while in glands from pharate pupae it was maximal at pH 3.5. However, such protease activity was not detectable in the haemolymph which suggests that there was no movement of protease from the salivary gland into the haemolymph or vice versa. In the present work with *Calliphora*, pepsin-like protease activity was found in the glands and in the haemolymph. The similarity between the curve for protein synthesis (Fig. 5) and that for protease activity (Fig. 11), both being near maximum in 6 to 7 day larvae, and the incorporation of radioactivity into protein behaving electrophoretically as protease, indicate that protease is synthesized in the gland. It is suggested that the fall in protease at the R.O. stage is the result of the gland no longer synthesizing the enzyme.

Protease activity in the haemolymph was highest in 5 day larvae after which it fell steadily over the remainder of the instar (Fig. 13). As the pH of blowfly larval haemolymph is 7.4 and as at this pH protease activity was not detectable (Fig. 10) then, presumably, the protease is not active in the haemolymph but is simply
stored there until it is sequestered by the fat body. The rapid rise in the level of protease in the fat body during the late third instar (Fig. 14) is more likely the result of the enzyme being sequestered from the haemolymph than to it being synthesized in the fat body, for at this time the overall rate of protein synthesis in the fat body is at its lowest level (Price, 1966). Furthermore, it was demonstrated that 14C-labelled proteins synthesized by, and released from, the salivary glands were sequestered by the fat body where over half the total 14C activity was associated with a 600 g precipitable fraction (Fig. 15). No 14C activity was detectable in the supernatant of cold perchloric acid extracts of any of the centrifugal fractions but only in the protein precipitate indicating that the 14C protein was not degraded after it entered the fat body. The high level of acid phosphatase in the 600 g precipitate suggests that this is a lysosome-rich fraction and as much of the 14C-labelled protein is also present in this fraction, then it is possible that the protein has been taken up by lysosomes the interior of which would provide a favourable acid-pH microclimate for protease to act.

That salivary glands in early third instar larvae secrete saliva via their duct is not disputed for when glands from 4 day larvae are dissected under Ringer's medium a slightly viscous secretion can be observed flowing out of the cut end of the common duct. On the other hand, in older (7 day) larvae, such a secretion is hardly discernible. The salivary gland would not be unique if its rôle changed during larval development for a similar phenomenon is exhibited by the fat body which in early third instar larvae synthesizes and releases protein into the haemolymph while in late larvae it sequesters protein back from the haemolymph (Price, 1973b). By functioning as a gland of internal secretion and releasing hydrolytic enzymes into the haemolymph, these being subsequently used by other tissues at the time of their lysis, the salivary gland would be playing a much greater rôle in the preparation of the larva for metamorphosis than had previously been thought.

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