INGESTION AND DIGESTION IN ADULT BLACK-FLIES (SIMULIIDAE)

A STUDY OF INGESTION AND DIGESTION, EMPHASIZING THE PERITROPHIC MEMBRANE AND DIGESTIVE ENZYMES IN ADULT SIMULIIDS (DIPTERA) FED BLOOD, BLOOD-SUCROSE MIXTURES AND SUCROSE

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TITLE: A study of ingestion and digestion, emphasizing the peritrophic membrane and digestive enzymes in adult simuliids (Diptera) fed blood, blood-sucrose mixtures and sucrose.

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SCOPE AND CONTENTS:

The blood and sugars, on which adult black-flies fed, stimulated the buccal sensilla so that blood went directly to the midgut and sugars to the crop. Agglutinin and anticoagulant were found in the salivary glands of female simuliids.

The sequence of peritrophic membrane formation and disappearance are discussed in relation to blood digestion.

Digestive enzymes, such as that of trypsin and invertase activity occurred in the midgut of both sexes of several black-fly species unfed or fed on sucrose, but an increase in enzyme activity was stimulated in females by a blood meal. A weak amylase-like activity was found in the midgut, but the activity was not stimulated by blood feeding. A greater amylase activity was found in the residual fluid (haemolymph). Trypsin activity in females, but not in males, increased also after feeding blood-sucrose mixtures. Black-fly invertase promoted oligosaccharide synthesis. Trypsin, amylase and invertase showed pH optima of 8.4, 6.5 and 6.2 respectively.

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INTRODUCTION

Bloodsucking simuliids, which occur throughout the world, are important vectors of disease organisms (Blacklock, 1926a,b; Skidmore, 1932; Twinn 1933; Steward, 1923) or are themselves of considerable economic importance due to the vicious bites which they inflict upon man (Sagard, 1923) and other animals, particularly livestock (Riley, 1887; Lugger, 1896; Cameron, 1918).

The literature includes only scanty reference to the studies of digestion in adult black-flies. Brues (1946) believed that saliva of a black-fly contained a powerful proteolytic enzyme which caused a minute haemorrhagic spot after the bite of the fly. This is contrary to the situation in other bloodsucking Diptera examined, as no proteolytic enzymes were found in the salivary glands of Glossina (Wigglesworth, 1929), Chrysops (Wigglesworth, 1931), Anopheles quadrimaculatus Say (Metcalf, 1945), Aedes aegypti (L.)(Fisk, 1950) and Stomoxys (Rostom and Gamal-Eddin, 1962). Wanson (1950) was the first to report on digestive enzymes in the midgut of a black-fly species. He mentioned briefly that Simulium damnosum Theob. had a weak amylase activity, but showed strong activities of tryptase and peptidases at alkaline pH, capable of digesting small fragments of coagulated yolk in vitro. While dissecting blood-fed black-flies to study ovarian development, Rubtzov (1958) gained an impression that the epithelial cells of the anterior part of the midgut secreted digestive enzymes only at the moment the blood entered the gut. This was postulated because of an intense granular secretion in the anterior midgut soon after a blood meal. However, such a granular secretion was reported by Cox (1938) in the midgut of unfed females

of S. jenningsi Mall.

Formation of a peritrophic membrane (PM) was observed by Lewis (1950; 1953) when studying S. damnosum and its relation to the parasitic disease, onchocerciasis. In flies killed during the ingestion of blood, no PM was observed but the membrane enclosed the whole blood mass half a minute following feeding. One to three days after the fly fed on blood, its PM broke and most of the blood mass disappeared.

From this résumé it is clear that until now only a limited insight into the physiological mechanism of digestion has been gained in simuliids. Because the family Simuliidae is of considerable economic importance, it seems worthwhile to gain a greater understanding of feeding and digestion in black-flies. This might help to elucidate host preferences and disease transmission by simuliids, in addition to the pure biological interest.

Therefore, the present studies were conducted to investigate the mechanism of a food ingestion and digestion in simuliids, with special emphasis on digestive enzymes and the formation of the peritrophic membrane which is closely related to blood feeding. The first part of the study was devoted to the switching mechanism by which ingested food goes to the crop or to the midgut, to the location of taste receptors which might regulate the switching mechanism, and to the function of salivary glands. Secondly, after the ingestion of blood by the fly, the formation and disappearance of the peritrophic membrane and the process of blood digestion were elucidated by morphological and histological studies. Finally the presence and characteristics of digestive enzymes, particularly trypsin, pepsin, amylase and invertase, in different tissues and organs of the black-fly and the change in the activity of each enzyme following adult emergence and in relation to different foods were investigated.

Although Simulium venustum Say, which is mammalophilic and the most troublesome black-fly species in Ontario, was chosen as the main experimental animal, other mammalophilic, ornithophilic, or non-bloodfeeding simuliids and mosquitoes were also employed for comparison or for other experimental purposes.

LITERATURE REVIEW

In general it is believed that the digestive enzymes in insects are adapted to the diet on which the insects feed in nature. Omnivorous insects, like the cockroach, secrete proteinase, lipase, amylase, invertase and maltase (Wigglesworth, 1927; Swingle, 1925). In the blowfly, feeding largely on sweet substances, proteinase activity is relatively weak, while amylase and invertase are active (Wigglesworth, 1929). On the other hand in Glossina, feeding exclusively on blood, proteinase activity is high, but carbohydrases are absent except for a very weak amylase activity in the midgut region (Wigglesworth, 1929). However, Chrysops (Wigglesworth, 1931) and Aedes aegypti (Fisk, 1950; Fisk and Shambaugh, 1954), which feed on both blood and nectar, occupy an intermediate position and have a strong proteinase and invertase activity, but a feeble, or no, amylase activity in the midgut. These observations presumably refer in each case to unfed insects and feeding may change the picture in some species (Thomsen and Møller, 1963).

Proteolytic Enzymes in Insects

Trypsin-like enzyme

Many species of insects have been studied for their proteolytic enzyme activity. The terms "protease", "proteinase" and "proteolytic enzyme" have been used synonymously in the literature; proteinase will be the term employed in this literature review. Reports indicate that one of the enzymes frequently found in these insects is a proteinase, which is usually active at neutral or alkaline pH, thus resembling vertebrate trypsin more than other known proteinases (Powning et al, 1951; Lin and

Richards, 1956).

Wigglesworth (1928), one of the earliest investigators of proteolytic enzymes in insects, compared the proteinase of the American cockroach with the human pancreatic trypsin and reported that the two enzymes were similar in (a) the product of digestion, (b) the relative production of "free acidity" and "formaldehyde activity" during digestion, (c) the effect of salts, and (d) their action upon proteins only on the alkaline side of their isoelectric point. The enzymes differed in that, with a given protein, the range of activity of the cockroach proteinase extended further in the acid direction, as evidenced by the pH acitivity experiments with different substrates. The theoretical significance of this fact has been attributed to the nature of the substrates used.

Increased secretion of proteinases stimulated by feeding in insects was first observed by Schlottke (1937ab). In a predacious ground beetle, Carabus auratus, and in Tettigonia cantans, he observed the sharpest increase in trypsin-like activity within the first hour after feeding. In Blattella germanica L., Day and Powning (1949) mentioned, that proteinase was still present in the midgut after three days of starvation, but that the enzyme increased in concentration when the insect was fed, irrespective of diet. The proteinase in the midgut of Blattella, however, was depleted when the roach was fed a diet which contained the specific substrate of the proteinase and recovery to the original level was fairly slow. Dadd (1956) reported the spontaneous occurrence of proteinase activity in Tenebrio after a moult and adult emergence, and an increase in activity in response to feeding in the active larva and the mature adult. Even water or damp cellulose feeding was effective in increasing the secretion in the adult. He further showed that although the proteinase accumulated in the midgut tissue of starved

Dytiscus, within an hour after feeding it was largely discharged into the crop. Proteinase activity reappeared in the midgut tissue a few hours after feeding, but remained low as long as the crop contained the undigested material.

Studying the distribution of digestive enzymes in various regions of the alimentary canal of the desert locust, Locusta migratoria L., Khan (1963a) found that the caeca showed a proteinase activity 4 times higher than the midgut, in both males and females. The proteinase activity in females, however, was higher than that in males. Later she (1963b) showed that there was a difference between the fed and unfed locusts of both sexes; proteinase activity was reduced to half in starved females. She found that the amount of the enzyme in the tissue was small as compared with that in the lumen. Thus she concluded that when enzyme was produced in the tissue, it was rapidly discharged into the lumen.

Fisk (1950) found that the proteinase activity of unfed females of A. aegypti was insignificant, while a measurable activity was found in the midgut from the females, which were permitted a partial blood meal a few hours prior to dissection. The presence of blood in the midgut apparently stimulated the proteolytic activity but neither the feeding itself nor the distension of abdomen was responsible for this stimulation. Fisk and Shambaugh (1952) investigated further the proteinase activity in relation to feeding in A. aegypti. The proteinase activity of fed females was found to drop in 5 min. to below the original level characteristic of unfed females. This drop was followed by an equally rapid increase for the first few hours which gradually decreased again until 48 hr., but did not reach the original value of unfed insects. On feeding sugar solution, there was an initial

two-fold rise in the proteinase activity during the first hour, but in 2 hr. the activity had dropped to the original value. Shambaugh (1954), in his studies of proteinase stimulation, found that the effect of sheep erythrocytes in a relatively inert medium produced a proteinase activity somewhat greater than the value in unfed flies. On testing the dialyzable and non-dialyzable portions of the blood for the stimulation of proteinase activity, he found that the more important stimulatory factors were in the non-dialyzable plasma protein, namely, fibrinogen, albumin, and gamma globulin of the blood meal when these were present in the diet at a concentration of 0.6%. He suggested a positive correlation between the amount of blood ingested by female mosquitoes and the subsequent proteinase activity of their midguts.

By judging protein content of the midgut, Gooding (1966b) observed that the rate of digestion was faster in A. aegypti than Culex fatigans Wied., but the decline in the protein content of the midguts during digestion of the blood meal was not markedly affected by photoperiod, or by the blood or mosquitoes being infected with Plasmodium gallinaceum. Gooding (1966a) studied the proteinases in the midguts of A. aegypti and C. fatigans and found that those of the two species had similar properties. These proteinases showed a small peak of activity near pH 5.5, but the greatest activity was found in a broad peak ranging from pH 8 to 10, with a temperature optimum near 46-50°C. A haemoglobin preparation was much more easily hydrolysed than albumintor gamma globulin by the proteinases. He also found that the proteinases were inhibited by serum from mammals and malarious chicks.

Champlain and Fisk (1956) studied a proteinase activity in the stable-fly and found the optimum pH for the hydrolysis of two substrates, azo-casein and azo-albumin, to be 7.8 and 7.9 respectively. Proteinase

activity, following a meal of sucrose, showed a slight increase for about 1 hr., but this activity quickly declined and remained close to the original value. For blood ingestion, however, the enzyme activity showed gradual increase for a longer period, reaching maximum proteinase activity about 13 hr. after feeding.

Langley (1966 a,b) conducted a series of studies with Glossina to elucidate the regulation of the production of those enzymes concerned with the digestion of the blood meal. There was a linear relationship between midgut proteinase activity and meal size 24 hr. after the tsetse flies fed on whole blood. A similar relationship was observed in flies after feeding on a series of dilutions of whole blood in saline, indicating that the volume of fluid ingested, but not the quantity of blood in the meal, controls the appearance of the active enzyme in the midgut. However, at least some blood must be present in the meal before active proteinase could be produced, because no increase in enzyme activity was obtained after the flies ingested saline alone.

The distribution of digestive enzymes in the alimentary canal of larvae of six species of scavenger flies of medical and veterinary importance was studied by Rockstein and Kamal (1954). They found a trypsin-like enzyme in the midgut of all species studied but not in other tissues, such as salivary glands and crop. Kamal (1959) extended the work on distribution of digestive enzymes and made comparative studies of digestive enzymes of 13 species of families Calliphoridae and Sarcophagidae (Diptera). Both papers are of considerable importance for understanding the distribution of trypsin-like enzyme in Diptera.

Wagner et al (1961) extracted and partially purified two proteolytic enzymes derived from aqueous homogenates of whole adult mosquitoes, A. aegypti.

In either case the increased activity resulting from purification was never greater than 10 to 20 times that of the original extract. One enzyme was a proteinase acting upon urea-denatured haemoglobin as a substrate, while the other resembled an esterase in its action on benzoyl-L-arginine ethyl ester. Partial purification of a proteinase was also made from homogenates of the larvae of the clothes moth, Tineola bisselliella Hummel, by Powning and Irzykiewicz (1962a). The purification resulted in a 400-fold increase of specific activity over the initial water extract. But when compared with crystalline trypsin, the purified Tineola enzyme had an optimum pH of 9.8 and possessed a distinct keratinase activity, while the latter had a pH optimum of 8.0 and no keratinase activity (Powning and Irzykiewicz, 1962b). The Tineola enzyme also had a higher specific activity on casein than the sample of crystalline trypsin.

Birk et al (1962), during research on the factors affecting growth of Tribolium castaneum (Herbst), T. confusum Duval and Tenebrio molitor L., subjected midgut homogenates of these beetles to soybean trypsin inhibitor with haemoglobin as a substrate, and observed the change in the proteolytic activity in vitro. The soybean trypsin inhibitor partially inhibited proteolysis in larvae of this mealworm (Tenebrio). For midgut as well as whole larval homogenates of these larvae, the optimum conditions for total in vitro proteolytic activity for midgut were pH 6.2-6.4, with 0.5% casein as substrate. Powning et al (1951) reported the pH optimum for the enzyme in the mealworm to be slightly alakline, i.e. pH 8.0 with gelatin as substrate. Smith et al (1964) reported that 5-fluorouracil (FU), which is generally known as an antimetabolite, inhibited oviposition in house-flies

screwworm-flies and fruit-flies, and Akov (1965) studied the effect of FU on the rate of oocyte growth and blood digestion in A. aegypti females. The results showed that FU inhibited the midgut proteinase production and ovarian development in vivo but it did not inactivate the proteinase in vitro. The extent of inhibition in vivo was proportional to the amount of FU and the time interval between FU feeding and the blood meal. When soybean trypsin inhibitor was used, however, it inhibited the proteinase activity both in vitro and in vivo. The results of feeding soybean trypsin inhibitor indicated that the inactivation of proteinase in vivo sufficed to inhibit ovarian development.

Thomsen and Møller (1963) studied the influence of neurosecretory cells and of corpus allatum on intestinal proteinase activity in the adult Calliphora erythrocephala Meig. They found that the development of the enzyme(s) was controlled by the medial neurosecretory cells (m.n.c) of the brain, and that the mean proteinase activity of females deprived of their m.n.c. amounted only to one-quarter to one-third of the values for the control flies. The corpus allatum hormone was found to have a minor effect on the proteinase activity. Thomsen and Møller (1963) also found that the proteinase activity of the adult Calliphora female, measured on the first few days after emergence, was highly influenced by the diet, the activity in females fed on sugar, water and meat being much higher than that in females fed only on sugar and water.

Proteolytic enzymes were demonstrated in tissues other than midgut of insects. Lichenstein (1947) observed the cleavage of casein at pH near 8.0 with glycerol extracts of the eggs of Bombyx mori L. only a few days before hatching. In 1954, Kuk-Meiri et al, studying proteinases in an insect egg, noted that there was a possible correlation in time between the

appearance of proteolytic enzymes and certain stages of the development of the eggs of Schistocerca gregaria Forskol. Later Shulov et al (1957) made similar observations with eggs of Locusta migratoria migratoriodes Reiche and Quirmaire. In no case was hydrolysis of casein or leucylglycylglycine obtained with freshly laid eggs. However, a distinct hydrolysis of casein was obtained with batches of eggs in developing stages. This action increased toward the end of development. They found at least two kinds of endopeptidases, having pH optima % 7.8 and 5.6 in Locusta eggs. From these results, it was concluded that the appearance of a proteinase activity at alkaline pH values was connected with the formation of the digestive system in the developing eggs. Birk et al (1962) found no substantial proteolytic activity in eggs of Tenebrio which were dissected from females and which were, therefore, at very early stage of development.

The presence of proteolytic enzymes in moulting fluid has been reported in Bombyx mori (Hamamura et al, 1940) and Cecropia silkworms (Passonneau and Williams, 1953). The latter authors reported that early moulting fluid obtained from the moth pupae during the first two weeks of adult development had no significant proteolytic activity. In constrast, the late moulting fluid during the third week of adult development showed considerable enzyme activity. The proteinase activity of 0.2 ml of the moulting fluid was approximately the same as that of 0.3 ml of 0.45% trypsin. The proteinase and chitinase in the moulting fluid are believed to hydrolyse the protein and chitin respectively in the overlying pupal endocuticle. Passoneau and Williams (1953) obtained no proteinase from the insect's blood at any stage of adult development, and indicated that the physicochemical and enzymatic properties of moulting fluid differed from those of blood.

The trypsin-like enzyme from the midgut of adult Stomoxys calcitrans L. was found to include at least three substances (Patterson and Fisk, 1958). Similarly Patel and Richards (1960) found that the midgut of Musca domestica L. contained three proteolytic enzymes, each having different substrate specificity and hydrolyzing gelatin optimally at different pH values, and yet differing in electrophoretic mobility from mammalian trypsin and pepsin.

Pepsin-like enzyme

Enzymes acting like pepsin in a strongly acid medium are generally believed to be lacking in insects. However, the presence of a pepsin and a cathepsin in addition to a trypsin was claimed by Greenberg and Paretsky (1955) in all three larval stages of the house-fly, on the basis of pH optimum of the enzymatic reaction. A pepsin-like enzyme has been also found in larvae of the stable-fly, Stomoxys calcitrans (Lambremont et al, 1959) and the pH optimum of the enzyme was determined to be 2.4. Fraser et al (1961) reported that the intestinal proteinase of adult females of Calliphora vomitoria L. had two peaks of activity, a high one at pH 3.0 and a lower one at pH 8.0.

Carbohydrases in Insects

The considerable literature on carbohydrases in insects has been reviewed by several authors (Day and Waterhouse, 1953; Waterhouse, 1957; House, 1965; Wigglesworth, 1965).

Amylases

In general, amylases have been found in most insects whose diets contain polysaccharides in addition to other nutrients. In omnivorous cockroaches (Swingle, 1925; Wigglesworth, 1927; Day and Powning, 1949; Fisk and Rao, 1964), mealworms (Fraenkel, 1955; Applebaum et al, 1966; Krishina and Saxena, 1962), phytophagous Orthoptera (Evans and Payne, 1964), larval

Lepidoptera (Shinoda, 1930; Stober, 1927; Schlottke, 1944) and omnivorous Diptera (Fraenkel, 1940; Hassett, 1948; Evans, 1956; Webber, 1957), active amylase(s) was(were) detected in the region of the midgut.

Amylase is also frequently found in the salivary glands of many insects. Strong amylase activity is present in the saliva of cockroaches (Wigglesworth, 1927; Banks, 1963), Calliphora (Wigglesworth, 1929, Evans and Payne, 1964), omnivorous Musca (Rostom and Gamal-Eddin, 1962) zoosaprophagous Wohlfartia trina (Rostom and Gamal-Eddin, 1962) and plant-sap feeders e.g., aphids (Bramstedt, 1948).

Two types of amylases have been identified in plants and animals. The exoamylases are able to attack the polysaccharides only from the non-reducing outer chain ends and to split off maltose from the straight chains of glucose units. This slowly affects the colour reaction of the polysaccharides with iodine. These enzymes are also called saccharogenic or β-amylases. Endoamylases attack the glycosidic linkage of the interior of the polysaccharide chains, breaking them into oligosaccharides. These compounds, although large enough to be nonfermentable, are of such low molecular weight that the solution rapidly loses the colour reaction with iodine, but maltose and glucose are only produced slowly. These enzymes are also called amyloclastic or α-amylases (Gilmour, 1961).

The two types of amylases have yet to be separately investigated in insects. The amylase of cockroach was inactivated by dialysis and was reactivated by the addition of chloride ion in the same way as is the α-amylase of human saliva (Wigglesworth, 1927; Day and Powning, 1949). However, certain differences were reported. The pH optimum for cockroach amylase was 6.0, while that for human ptyalin was 6.5. Amylase from the midgut of the mealworm resemble α-amylase in its being activated by Ca^{++}

and Cl^-, but resembles β-amylase of in being inhibited by Hg^{++}, Cu^{++}, and ascorbic acid, which do not affect α-amylase (Applebaum et al, 1961).

When cockroach amylase was heated in a boiling water bath, a slight activity still remained after 2 min, but none after 5 min (Day and Powning, 1949). Mealworm amylase was stable at least for 20 hr at 5°C, but the enzyme lost 75% of its activity on dialysis at this temperature.

Day and Powning (1949) demonstrated that amylase activity was high in cockroaches fed on a normal diet (cut potato, bran mash and water), but was considerably reduced by starvation for three days. Feeding gelatin or sucrose also slightly reduced amylase activity.

Krishna and Saxena (1962) observed that all carbohydrases in the midgut of both the larvae and adult of Tribolium castaneum showed optimum activity at 5.5. The various carbohydrases in this insect differed from one another in the following order according to their activity: amylase > invertase > β-glucosidae > α-galactosidase > β-galactosidase.

Invertases

In 1924, Nelson and Cohn made a careful comparison between the invertases of honey and of yeast. They found certain differences in the kinetics of the reaction in relation to substrate concentration, but the optimum concentration of sucrose for both enzymes was about 4%. The pH optimum ranged from 3.5 - 5.0 in yeast and from 5.5 - 6.3 in honey invertase. Wigglesworth (1927) compared cockroach invertase with yeast and human jejunal invertases. The pH-activity curve for cockroach invertase showed an optimal range from 5 to 6.2, which was intermediate between the ranges for yeast and human invertase. The cockroach invertase was partially inhibited by 1% sodium chloride, 20% glycerol and 0.0001 N silver nitrate, and completely inhibited by 0.001 N silver nitrate.

Day and Powning (1949), studying the sites of enzyme production in Blattella and Periplaneta, observed that invertase activity decreased slightly from anterior to posterior in the midgut of both species. Practically no invertase activity was present in the crop. Different foods caused no change in invertase activity in the midgut cells, but the activity in the midgut contents decreased immediately after starch or sucrose were fed. However, both of these foods increased the enzyme activity during the following 1-hr testing period, compared to the first 10 min after feeding. On the other hand, in Tribolium castaneum, invertase activity in the midgut remained almost the same regardless of time of feeding or period of starvation of the insects (Krishna and Saxena, 1962). It was suggested, therefore, that in this species concentration of invertase in the midgut did not depend upon the stimulus provided by the ingestion of food.

In female mosquitoes, A. aegypti, significant invertase activity was detected (Fisk and Shambaugh, 1954). The enzyme activity in diverticula was lower than in the midgut, and the activity in the diverticula decreased after feeding on sugar or blood. A definite increase in enzyme activity, however, occurred in the midgut after blood feeding, while with sucrose feeding there was little change.

Khan and Ford (1962), studying sucrase (invertase) in Dysdercus fasciatus Sign, believed that feeding apparently provided the stimulus for sucrase production and the entire midgut reacted as one unit, and they suggested this stimulus was hormonally transmitted, since the gut appeared to be poorly innervated.

Two types of invertases have been known in different organisms. One is β-h-fructofuranosidase, which hydrolyses sucrose by attacking the

fructose moiety of the molecule. The same enzyme is also capable of hydrolysing raffinose, and in this case also, it is the fructose part of the molecule which is attacked. However, this enzyme cannot act upon melezitose, because the fructose moiety of the molecule is blocked by glucose. The second type, known as glucosucrase or α-glucosido-invertase, is an α-d-glucosidase, which can hydrolyse sucrose by attacking the free glucose end of the molecule. It can also hydrolyse melezitose by attacking the unsubstituted glucose moiety, but this enzyme cannot act upon raffinose, where the glucose moiety is blocked by galactose (Neuberg and Mandl, 1950; Gilmour, 1961).

Invertases in insects are generally known to belong to the α-glucosidase type (Waterhouse, 1957; House, 1965), although the β-fructofurnanosidase has also been reported. Evans (1956) obtained two optimum pH values for the hydrolysis of sucrose by midgut extracts of Calliphora, and he suggested that the sucrose molecule was attacked by an α-glucosidase and a β-fructosidase, both present in the extract but with different pH optima. When sucrose was replaced by an α-methyl glucoside substrate, Evans showed that the enzyme also hydrolysed the substrate, with an optimum value corresponding to the lower of the two values obtained with the sucrose substrate. This was thus assumed to be the true optimum of the α-glucosidase since α-methyl glucoside is unaffected by a β-fructosidase. Two similar pH optima for the hydrolysis of sucrose were obtained by Khan and Ford (1962) from the midgut extracts of Dysdercus fasciatus, but the gut extracts were unable to hydrolyse α-methyl glucoside and the presence of a β-fructosidase was unconfirmed. However, the invertase in the gut of Dysdercus koenigi hydrolysed sucrose, raffinose and melezitose, which indicated that the insect secreted both α-glucosidase and β-fructosidase. Similarly both types of invertases

were demonstrated in the digestive tract of cockroaches (Ehrhardt and Voss, 1962; Banks, 1963).

The invertase from yeasts and moulds is regarded as a transfructosidase which builds up a series of oligosaccharides by the addition to an acceptor (usually sucrose) of successive fructose units, whereas honey and nectar invertase is transglucosidase since this enzyme synthesizes a comparable series with glucose units (Myrback, 1960; Gilmour, 1961).

Considerable information is available on the synthesis of oligosaccharides by the action of insect invertases. The common oligosaccharide found in the reaction mixtures after the action of honey invertase on sucrose was a trisaccharide with the structure of glucose-glucose-fructose (White and Maher, 1953). Gray and Fraenkel (1953; 1954) independently discovered the same sugar, which they called fructomaltose, in the honeydews of Aphis spiraecola Patch, Pulvinaria vitis L., Pseudococcus citri Risso and the excreta of the blow-fly, Phormia regina Meig. Wolf and Ewart (1955) found a similar oligosaccharide (also maltosucrose and maltotriosucrose) from the excreta of Coccus hesperidum L., and Baron and Guthrie (1960) identified three oligosaccharides (glucosucrose, maltosucrose and maltotriosucrose) from the honeydews of Myzus persicae Sulz. Saxena and Bhatnagar (1961) reported that in the lygaeid, Oxycarenus hyalinipennis Costa, a trisaccharide was produced by the action of gut invertase on sucrose *in vivo* but not *in vitro*.

The above-mentioned oligosaccharides and invertases have been derived almost exclusively from insect excretory products which have been subjected to an unknown degree of bacterial action. However, Duspiva (1953, 1954) demonstrated that the invertase from the gut of aphids synthesized oligosaccharides *in vitro*, and these were also found in the excreta of the aphids.

Srivastava and Auclair (1962) similarly reported the findings of oligosaccharides from the incubation mixtures of sucrose and gut extract of aphid, Acyrthosiphon pisum (Harr.).

Peritrophic Membrane in Insects

It is well known that the epithelial cells of the insect midgut are endodermal origin. Therefore, unlike the foregut and hindgut, the midgut lacks a chitinous cuticle. However, the food in the midgut is kept from direct contact with the epithelial cells by being enclosed in a delicate single or multilayered sheath known as the peritrophic membrane (PM).

Until recently it was generally believed that there were two types of PM which were formed in two different ways. 1.) The first type occurred in Coleoptera, Hymenoptera, many Diptera and some other insects (Waterhouse, 1957; Wigglesworth, 1965). The PM of these insects consisted of thin independent and loosely combined concentrated lamellae. It is formed by periodic delamination through secretion from the midgut cells. These cells usually have a striated border on which new sheet forms and by continuous secretion these sheets are separated from one another. When the newly formed lamellae in the bee (Dehen, 1933) were stained, they often showed polygonal areas corresponding to the cell surfaces by which they were laid down. 2.) The second type was found in Dermaptera, some families of Lepidoptera, many larval and adult Diptera, and adult Cyclorrhapha (Dixon, 1952; Waterhouse, 1953, 1957; Wigglesworth, 1965). The PM is secreted in viscous form by group of cells in a ring form of the proventriculus at the anterior end of the midgut; as the membrane substance passes through the narrow cleft between the proventricular wall and

the invaginated foregut it is pressed and solidifies to form a cylindrical peritrophic membrane consisting of a single uniform layer.

Besides these two distinct types of PM formation, the PM may have a double origin combining the features of the two types described above, and perhaps representing a stage in the evolution of the second type from the first (Wigglesworth, 1965). Such a dual origin of PM is reported in Calotermes, Bombyx mori, Tineola, larval aphids and Myrmica (Wigglesworth, 1965). Bloodsucking insects, such as adult Simuliidae (Lewis, 1950, 1953) and adult Culicidae (Stohler, 1957), are reported to produce from the whole midgut epithelium a viscous secretion, enveloping the gut contents entirely.

Although Stohler (1957), on the basis of his finding of the different origin and structure of the PM in A. aegypti, proposed a type 3 PM, Wigglesworth (1965) declined to divide the PM into three types, because with the increasing knowledge of origin of PM, type 1 and 2 actually merge into one another as described above.

The structure of the PM has been studied under the electron microscope (Mercer and Day, 1952; Waterhouse, 1953; Stohler, 1957). Electron-microscopic structure of the PM in different insects seems to be of two types; one with a distinct and regular network of fibrils (mesh); the other with a scattered, irregular arrangement of fibrils; both are coated with a protein film.

The chitin content of the PM was proven by several workers (Wigglesworth, 1929; Richards and Korda, 1948; Waterhouse, 1953). It was established that the PM had the same components as the inner layers of cuticle, i.e. a base of chitin, with protein incorporated into it (Wigglesworth, 1965). The membraneous chitin which appears to be a

mucopolysaccaharide is combined with protein (Gilmour, 1961).

It was believed that the PM protects the midgut cells from damage by the ingested hard foods, thereby performing the functions of mammalian mucus, material which is absent from the lumen of the insect midgut (Day, 1949; Waterhouse, 1957). However, the PM has recently been discovered in nectar- and blood-feeding insects which were formerly believed to be without a PM. The liquid foods of these insects would appear to have little or no roughage. Speaking of the higher Diptera, Zhuzhikov (1964) explains the protective function in a somewhat different manner. The protein food which is ingested by the insects forcefully attracts water and the swollen midgut contents stretch the PM. If there were no PM, all of the pressure, transmitted to the midgut walls, could lead to injury of the epithelium or even rupture of the walls. By resisting the pressure of the food masses, the PM protects the walls of the midgut from injury.

Other investigators suggested that the PM may serve as an ultrafilter (Gordon and Chambers, 1941; Schildmacher, 1950). The permeability of the membrane seems to vary somewhat from one insect to another, but it offers no difficulty for digestion, because digestive enzymes and the products of their action readily pass through it (Wigglesworth, 1929; Dehen, 1933). However, Zhuzhikov (1964) claimed that the PM cannot be completely likened to an ultrafilter. According to his study with house-fly, the PM is permeable in both directions only to the end products of food digestion, such as amino acids, mono- and disaccharides, etc., and the PM allows the digestive enzymes to pass only into the PM cavity but not out of the membrane cavity. He thus suggested that the two- or three-layered PM with

its layers of liquid colloids is a complex biocolloid system which selectively, and presumably actively, passes various substances.

MATERIALS AND METHODS

Collection of Specimens

Most of the adult black-flies used were reared from larvae and pupae collected in the Algonquin Park, Ontario, from 1965 to 1967. The larvae were maintained in plastic containers (7.5 x 7.5 in.) in which water was agitated by air bubbles. Small amounts of bakers' yeast were added regularly as food and the containers were kept at 10 to 15°C. Emerged adults were held at 18-20°C in cardboard cylinders (3.5 x 4.5 in.), the top and bottom of which were covered with nylon screen. The bottom of the cylinder rested on water-soaked absorbent cotton in a plastic petri dish and a sugar cube was supplied on the top screen for the adults. Other black-flies were collected, engorged with blood, from different hosts (Table 1) at the Wildlife Research Station, Algonquin Park, Ontario, during the summers of 1965 to 1967. The ages and feeding cycles were unknown for the wild-caught adults. Most of the blood-fed flies were kept in the cardboard cylinders under the conditions described above, unless otherwise mentioned.

Most of the studies involved the mammalophilic black-fly, Simulium venustum Say. However, other simuliid species were used in certain parts of research as follows:

Prosimulium

*decemarticulatum (Twinn)

fuscum Syme and Davies

gibsoni (Twinn)

Cnephia

**dacotensis (Dyar and Shanon)

*invenusta (Walker)

mutata (Malloch)

Similium	Similium
*aureum Fries (mainly type A)	*quebecense (Twinn)
*croxtoni Nicholson and Mickel	*rugglesi Nicholson & Mickel
decorum (Walker)	verecundum Stone & Jamnback
*excisum Davies, Peterson & Wood	luggeri Nicholson & Mickel
*latipes (Meigen)	vittatum Zetterstedt

*Ornithophilic and **non-bloodsucking species. All others mammalophilic species.

In a few aspects of the study mosquitoes were also involved. Adults of Aedes aegypti (L.) came from an inbred colony in this laboratory and females of Culex pipiens var. pipiens L. were collected in the autumn in buildings of McMaster University.

Induced Feeding Techniques

Before feeding the flies on blood substances in captivity, the sugar was removed from the cage overnight or for 24 hr. Whole blood[1] or a blood-sucrose mixture was soaked in the cotton and which was then placed on the top screen of the cylinder, or alternatively the blood substances were directly supplied spread in small droplets on the top of the screen. Membrane feeding techniques (Tarshis, 1958; Collins et al, 1964) were tried without success.

Preparation of Homogenates

To prepare various tissues of the fly for assays, flies were immobilized by chilling in a deep freezer for 10-20 min and then dissected in cold

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1. Citrated human blood was obtained from the Canadian Red Cross, citrated or or oxalated cow blood from Essex Packers, Hamilton, and the other types of blood from animals in the laboratory. Heparin (0.2 mg/ml), sodium citrate (5mg/ml blood) or sodium oxalate (0.2 mg/ml blood) was used in blood collections in the last instance.

buffers[2] or distilled water under a dissecting microscope at 50x magnification. The various tissues to be studied were separated and placed in small vials (10 x 3.5 mm), a number of similar tissues being pooled.

Whole flies, carcasses[3] or ovaries with fully grown eggs were homogenized in the appropriate buffer using a pyrex tissue grinder and after centrifuging at 2500-3000 rpm for 10 min, additional buffer was added to the supernatant to make up a volume of 0.5 ml or 1.0 ml, depending upon the number of tissues used. On the other hand, the midguts, hindguts, crops, salivary glands[4] or Malpighian tubules were macerated with fine needles under the dissecting microscope, and after making up to 0.5 ml or 1.0 ml by adding buffer, the suspensions of those and residual fluids[5] were used as enzyme sources without centrifugation.

For proteinase tests, all of the homogenates and suspensions were immediately stored in a deep freezer (-30°C) until needed. However, carbohydrases were assayed in freshly homogenized extracts.

All glassware coming into contact with the enzyme sources was washed twice with detergent, rinsed in running tap water for 5-10 min, acid-cleaned, rinsed again in running tap water for 3-5 min, and then at least three times with glass-distilled water.

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2. For trypsin, M/5 tris aminomethane/HCl buffer at pH 8.0.
   For pepsin, M/10 glycine/HCl buffer at pH 2.5.
   For amylase, M/15 sodium phosphate buffer at pH 6.5.
   For invertase, M/15 sodium phosphate buffer at pH 6.2.
3. Remainder of a fly after removal of alimentary tract and ovaries.
4. Salivary gland extracts were also made in 50 µl to 100 µl for microdeterminations.
5. Residual fluid consists mainly of haemolymph and to a lesser extent of other material which remained in cold buffer or water after removal of dissected tissues.

Determination of Trypsin-like Enzyme

Substrate for Trypsin Analysis

A synthetic substance, p-toluenesulfonyl-L-arginine methyl ester HCl (TAME)[6] was used as the substrate for trypsin analysis in this study because it had been shown to be a most specific and suitable substrate for determination of even traces of trypsin by several workers. Schwert et al (1948) found that TAME is rapidly hydrolyzed by trypsin (900x faster than benzoyl-L-argine amide (BAA)), and that it does not undergo spontaneous hydrolysis over a wide pH range, nor is it hydrolysed by chymotrypsin. Later Schwert and Eisenberg (1949) demonstrated that the products are not competitively inhibitory. Considering the advantages mentioned above, Hummel (1959), using TAME as substrate, was able to determine traces of trypsin in the presence of larger amounts of chymotrypsin using a spectrophotometric method.

Siegelman et al (1962) was first to apply TAME to determine trypsin in normal and diseased human serum, and developed a useful colorimetric method. Rao and Fisk (1965) adapted this method to investigate trypsin activity in the female Tampa cockroach.

Chemical Reagents

The following reagents were prepared for trypsin analysis:

Buffer; Boric acid-borax buffer 0.05M aqueous adjusted to pH 8.0.

Substrate; TAME[6] 0.25M prepared by dissolving 950 mg in 10 ml of water. This was prepared fresh every second day, and refrigerated between tests. Thus, the possibility of autohydrolysis was avoided.

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6. Nutritional Biochemicals Co., Cleveland, Ohio.

Calcium chloride buffer; 0.05M, aqueous adjusted to pH 2.5 with HCl. This was used as a diluent for the crystallized trypsin.

Trypsin[7]; For standard, the stock solution of 100 µg/ml was frozen between uses.

Trichloroacetic acid (TCA); 10% for terminating the enzyme reaction and 5% TCA for blank and for diluting the methanol standard.

Potassium permanganate; 2% aqueous, as an oxidizing agent.

Sodium sulfite; 10% aqueous, as a reducing agent.

Chromotropic acid[8]; For the working reagent, 200 ml of cold water was added to 100 ml of 2% aqueous chromotropic acid in a liter volumetric flask. To this 600 ml of cold, concentrated sulfuric acid was added slowly. When cooled to room temperature, the final volume was made up to 1000 ml with water.

Methanol standard; 0.003M methanol was prepared in 5% TCA.

All the reagents were stored in the refrigerator.

Procedure

The procedure for the determination of trypsin was slightly modified from the method of Siegelman et al (1962). Methanol released by hydrolysis of TAME by trypsin was oxidized with permanganate to formaldehyde which was measured colorimetrically by the use of chromotropic acid.

The trypsin activity of black-fly homogenates was assayed in duplicate. A 50 µl portion of homogenate was incubated with 0.8 ml borax buffer and 0.2 ml of the substrate in a test tube at 37°C for 1 hr. After the incubation, further reaction was prevented by adding 10% TCA. Following

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7. Crystalline, 2X, salt free, from General Biochemicals, Chagrin Falls, Ohio.
8. 4,5-dihydroxy-2, 7-napthalenedisulfonic acid, disodium salt, from Eastman Organic Chemicals, Rochester, N.Y.

centrifugation at 2500-3000 rpm for 5 min, 0.5 ml of the supernatant was treated with 0.1 ml potassium permanganate, decolorized by adding 0.1 ml of sodium sulfite and then mixed with 4.3 ml chromotropic acid. After the solution was heated for 15 min in a boiling water bath and cooled for 3 min in running water, the optical density at 580 mμ was determined against water in a "Spectronic 20" colorimeter. A blank was treated in same way except that the homogenate was added after the enzyme was inactivated by 10% TCA. Tubes containing only 0.5 ml of 5% TCA (a TCA blank) or 0.5 ml of stock standard (methanol standard) were introduced along with the test homogenate tubes just before the oxidation process. Optical densities of TCA blanks were deducted from experimental readings and corrected optical densities were read against the trypsin standard curve to obtain trypsin activity, and duplicate values were then averaged. Trypsin activity is expressed in terms of μg of pure crystalline trypsin per 10 flies (or tissues)/ml, unless otherwise stated.

Determination of Pepsin-like Enzyme

To determine pepsin-like enzyme activity, the methods of Champlain and Fisk (1956) and Lambremont et al (1959) were adapted. One tenth ml of homogenate and 0.9 ml of 0.1M glycine-HCl buffer (pH 2.5) were incubated with 0.25 ml of sulfanilic acid azoalbumin[9] substrate at 37°C for 2 hr. Unhydrolyzed protein was separated by centrifuging the incubated mixture for 10 min at 2500-3000 rpm. Optical density of the supernatant (the diazotized, colored amino acids or peptide fragments released in direct proportion to enzyme activity) was determined in a "Spectronic 20" colorimeter at 440 mμ. Controls consisted of reaction mixtures containing boiled

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9. Sigma Chemical Co., St. Louis.

homogenates, and also of reaction mixtures in which homogenate was replaced by buffer to determine autolysis and residual color of the substrate. The method was standardized using crystalline pepsin.[10]

Determination of Amylase-like Activity

To determine a starch-hydrolysing activity, the procedure, developed by Smith and Roe (1949) with the use of starch and iodine, was modified for microanalysis. The reaction mixture consisted of 0.1 ml of 0.5M NaCl, 0.5 ml of 1% buffered (pH 6.5) solution of boiled starch to which 0.4 ml of buffered homogenate was added. For small samples, the reaction mixture was modified to consist of 0.15 ml of the buffer, 0.05 ml of 0.5M NaCl, 0.2 ml of the starch solution to which 0.1 ml of the homogenate was added. The tubes, labelled as experimental (reaction mixture plus homogenate), control (reaction mixture plus homogenate boiled for 10 min) and blank (reaction mixture plus buffer), were given the same treatment and run in duplicate. Although toluene was used as a bacteriostat early in this investigation, it was omitted later when it was found to be unnecessary. After incubation for 30 min or 60 min at 37°C, the reaction was stopped by adding 0.5 ml N HCl (0.3 ml for small sample incubate). After adding 0.1 ml of 0.03% iodine solution (0.05 ml for small sample incubates), the whole mixture was diluted to 5 ml with water (2 ml for small sample incubates), and the resulting color read against the blank at 620 mμ in a "Spectronic 20" colorimeter. The value of the starch-hydrolysing activity was expressed as the difference between the optical densities of the control and the test mixtures.

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10. General Biochemicals, Laboratory Park, Chagrin Falls, Ohio.

Determination of Invertase Activity

Glucose oxidase-peroxidase system for invertase analysis

This system has been used broadly for accurate determination of glucose in blood, plasma, serum, urine and other biological fluids, and many procedures for the determination have been reported (Keilin and Hartree, 1948, 1952; Keston, 1956; Hugget and Nixon, 1957; Beach and Turner, 1958; Salomon and Johnson, 1959; Kingsley and Getchell, 1960; Washko and Rice, 1961; Saifer and Gerstenfeld, 1965; Weatherburn and Logan, 1966).

In this system, glucose is oxidized in the presence of glucose oxidase to gluconic acid and hydrogen peroxide. In turn, a chromogenic hydrogen donor, such as O-dianisidine (3.3-dimethoxy benzidine), is oxidized by the hydrogen peroxide, through the action of peroxidase, forming a colored substance that is stoichometrically related to the amount of glucose originally present. The overall simplified scheme of the reaction is as follows:

$$C_6H_{12}O_6 + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + C_6H_{12}O_7$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \text{oxidized chromogen}$$

Keilin and Hartree (1948) first demonstrated the usefulness of glucose oxidase for manometric studies of kinetics of reactions catalyzed by di- and polysaccharidases. Later, modifications of this system were introduced to assay hexokinase, alkaline phosphatase and a group of intestinal glycosidases (Sols and de la Fuente, 1957, 1961) and of intestinal disaccharidases (Dahlqvist, 1964).

Chemical reagents

Buffers; M/15 sodium phosphate buffer, pH 6.2

M/2 tris-aminomethane/HCl buffer, pH 7.0

Tris-glucose oxidase (TGO) reagent: From a prepared enzymatic glucose reagent, GLUCOSTAT,[11] the contents of the chromogen vial were dissolved in 1 ml methanol and shaken until clear, then 2-3 ml tris buffer were added. The contents of the glucostat vial were dissolved in another 4-5 ml of tris buffer. The two solutions were mixed and diluted to 100 ml with tris buffer.

Substrate concentration: A 0.056M solution of sucrose[12] in phosphate buffer. Toluene, about 1 ml/100 ml substrate solution, was added as preservative.

Standard glucose solution: 100 mg glucose was dissolved in 1000 ml distilled water saturated with benzoic acid. The solution was stable for months at room temperature.

Procedure

The procedure for the assay of invertase activity in simuliids was slightly modified from that of Dahlqvist (1964). In this method both α- and β-glucose react rapidly. The presence of moderate amounts of proteins do not interfere with the reaction of glucose (Dahlqvist, 1964).

Equal amounts (0.1 ml) of the homogenate and substrate solution were mixed in a small test tube (10 x 70 mm), and a small drop of toluene added. After incubation for 1 hr at 37°C, 0.8 ml distilled water was added, followed by immersion of the tube in boiling water for 10 min, to arrest further enzyme action. The tube was then cooled in water. A blank was treated in the same

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11. Worthington Biochemical Co., Freehold, N.J.
12. The British drug house Ltd., Laboratory Chemicals Div., Toronto.

way except that homogenate, boiled for 10 min, was used.

To determine the glucose in incubation mixtures, 0.5 ml of the solution mixture was transferred to another test tube (16 x 150 mm) and mixed with 3.0 ml of the TGO reagent. The tube was incubated as described above for development of color. At the same time a standard series, containing 0, 10, 20, 30, 40, 50 µg/ml of glucose was incubated. After the color had developed, the optical density was determined in a "Spectronic 20" colorimeter at 420 mµ, against reagent blank. All tests were run in duplicate. Glucose concentration was determined from the standard curve.

Invertase activity was obtained by the following formula (Dahlqvist, 1964):

$$\text{Disaccharidase activity (units/ml)} = \frac{(a - b).d}{n.\ 540}$$

where a = amount of glucose (µg) found in an aliquot of the incubated sample.

b = amount of glucose (µg) found in the corresponding blank.

d = extra dilution factor of the homogenate used for mixing with the substrate.

n = number of molecules liberated per substrate molecules hydrolysed.

540 = molecular weight of glucose (180) x incubation time (60 min)/20.

Units of activity = µ moles disaccharide hydrolysed/min.

Chromatographic Techniques

Paper partition chromatography was applied to separate substrate and products of the reaction during invertase analysis. With disposable

Sahli A/1 pipettes,[13] 20 µl of sample from the incubates were placed on filter paper (Whatman No. 1) along with the reference sugars and controls. After allowing the solvent, n-butanol-acetic acid- water (12 : 3 : 5 v/v) to run down the filter paper sheet for 24 hr, the chromatogram was dried and developed with benzidine - trichloroacetic acid reagents (Smith, 1958).

Determination of Anticoagulant and Agglutinin Factors

A pair of salivary glands was macerated in 5 to 15 µl isotonic saline under a dissecting microscope.

Anticoagulant activity was assayed by a micromodification of the clotting time determination (Biggs and MacFarlane, 1962; Hellman and Hawkins, 1964) in oxalated plasma or standardized normal human plasma.[14] To 10 µl of the plasma on a concave microslide is added 5-10 µl of the salivary gland extract. The slide was then placed on a test tube rack in a water bath at 37°C and 10 µl of M/40 $CaCl_2$ was added. The time taken for coagulation to occur was recorded.

For the agglutinin test, erythrocytes obtained from human, cow, chicken and duck were used. The cells, washed three times in physiological saline, were diluted to 5 to 10% in saline. To 5 µl of salivary gland extract on a microscopic slide, 10 µl of the diluted blood cell suspension was added. Agglutination of the blood cells by the extract was checked at 10 min intervals over a 30-min period at room temperature.

Chitosan Tests

Peritrophic membrans dissected from blood-fed female black-flies were placed in saturated aqueous KOH in a sealed capillary tube and heated at 150°C in an oven for 30 min. The undissolved fragments were washed with

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13. Clay-Adams Inc., N.Y.
14. DADE Reagents, Inc., Miami, Florida.

90% and 50% ethanol and then with distilled water before being tested for chitin with 0.2% iodine in 1% sulfuric acid (Wigglesworth, 1929, Waterhouse, 1953). The presence of chitin was indicated by the purple color after the addition of the iodine.

Histological Techniques

Because of the problem of sclerotized chitin in the adult blackflies, the following fixatives were tried: Carnoy and Lebrun's, alcoholic Bouin's, Zenker's, Sinha's and Gender's fixatives (Humason, 1960). Carnoy and Lebrun's and alcoholic Bouin's gave better sections than the others and were exclusively used in the work reported below.

The specimens (Table 1) were dehydrated as usual in a series of gradually increasing percentages of ethanol in water, and then in absolute ethanol, with final clearing in cedarwood oil. During dehydration, the wings and legs were removed from the specimens. After infiltration, most of the insects were embedded in tissuemat paraffin (60-63°C melting point), although some insects were processed with a double embedding method using methyl-benzoate-celloidin solution (Humason, 1960).

Serial sections were cut either longitudinally or transversely at 8-10 μ thickness. Because the stomach was full of blood or the ovaries full of yolk, the specimens were sometimes very brittle and shattered under the microtome knife instead of yielding coherent sections. This effect could be minimized by painting, before cutting, each section with a 0.5% solution of collodion in absolute ethanol and ether to prevent the crumbling (Lillie, 1965).

Delafield's haematoxylin and eosin were used routinely for staining the sections and Mallory's triple connective-tissue stain was occasionally used for the peritrophic membrane (Waterhouse, 1953).

RESULTS

I Morphology and Histology of Digestive System of Adult Simuliids

The alimentary canal of the female black-fly consists of a simple, straight tube which is divided into foregut, midgut and hindgut (Fig. 1). The chitinized foregut functions as a sucking pump in the cephalic region; mandibles, maxillae and hypopharynx play an important role in making a wound and the labrum and hypopharynx form a canal for the flow of blood into the cibarial pump. The heavily chitinized cibarial and pharyngeal pumps actively pump the blood into the oesophagus. In the thorax the thin-walled lightly sclerotized oesophagus, which extends from the pharyngeal pump, forms a weak expansion posteriorly. At the junction of the oesophagus with the midgut is a structure termed the proventriculus or cardia (Christophers, 1960)(Figs, 3, 4, 5). This is a thickened portion of the midgut, formed partly from the cardiac region of the midgut and partly from invagination of the oesophageal walls of the foregut. The component cells of the cardia completely lack a striated border (Figs. 3-5).

At the posterior end of the oesophagus just anterior to its junction with the cardia is the opening of the crop or diverticulum (see Figs. 3-5). The crop lies in the mid-line ventral to the midgut and dorsal to the ventral nerve chain, and extends posteriorly into the abdomen. The crop forms a sac which is thrown into innumerable small folds when it is collapsed (Figs. 6, 7), but when greatly distended, it occupies almost the whole abdomen. In a fresh preparation, the wall of the crop appears as a thin transparent membrane which is highly elastic. However, in histological sections the wall is seen to

consist of an inner layer of very thin epithelial cells with small nuclei and an outer muscular layer (Fig. 8). The crop is believed to serve exclusively for the storage of water and sugar solutions which are the only nutrient for males and which also are important for females which ingest such food in addition to blood.

The midgut or ventriculus is that part of the digestive tract between the proventriculus and the pyloric sphincter. The anterior half of the tubular midgut, which is situated in the thorax, is narrow and does not serve in storing and digesting blood, although after a full blood meal some blood remains in it until the blood volume in the posterior midgut or "stomach" decreases. Only the posterior midgut receives much blood and plays an active role in digestion. The posterior midgut shows considerable flexibility and when a full blood meal is taken, it expands to an almost ovoidal form. Both the anterior and posterior midgut consists of a single layer of columnar or cuboidal cells, each with a more or less centrally located oval to round nucleus and granular cytoplasm. On the outer surface of the basement membrane of the midgut, there is a network of longitudinal and circular muscle fibers, together with a rich supply of tracheoles. In fully engorged flies in which the posterior midgut is greatly distended, the epithelial cells become stretched and flattened and the nuclei lie with their axes at right angles to the gut lumen.

The hindgut begins at the posterior end of the midgut, and has a strongly sclerotized inner lining . Its anterior end is marked by a sphincter and four long Malpighian tubules open just behind this, so that they empty into the pyloric chamber of the hindgut, and their products are excreted through the anus.

The salivary glands are paired structures which lie in the antero-dorsal region of the thorax. Each gland consists of (i) an elongated, secretory portion, bent on itself at the mid-point in the form of a U, forming a distal arm and proximal arm, and (ii) a rounded portion which is connected with the proximal arm. The salivary ducts from the salivary reservoir extend ventrally each side of the oesophagus to unite beneath the sub-oesophageal ganglion in the head (Fig. 9). From the point of junction, the salivary duct expands, and its inner walls are sclerotized, forming salivary pump. Anteriorly, the salivary pump narrows and passes up a keel-like ridge on the ventral surface of the hypopharynx to open on the center of its upper surface.

The size of the salivary glands varies in different species and even in different specimens of the same species. The salivary glands of S. decorum females immediately after emergence from the pupae were clearly smaller than those of older females (Table 2).

II Ingestion of Foods by Adult Simuliids

(a) Induced Feeding of S. venustum with Whole Blood or Blood-Sucrose Mixtures

Laboratory breeding of successive generations of any simuliid species has generally been unsuccessful. This is partly because these species feed reluctantly in captivity. To induce captive S. venustum females to feed on hosts in large numbers has been unsuccessful. Because it was known that female simuliids under natural conditions feed on nectar and water in addition to blood, an attempt was made to feed them on blood-sucrose mixtures. S. venustum females were netted as they flew around the author in Algonquin Park, Ontario, in the early summer of 1966 and were

kept in the laboratory until used. When the contents of 10 crops were tested with Benedict's solution or were chromatographed, they were shown to contain fructose, glucose, sucrose and a small amount of oligosaccharides.

Whole blood or mixtures of saturated sucrose solution and citrated human, chicken, or duck blood were prewarmed to 40°C and provided for 3 hr to groupsof female flies which had been given only water for the previous 24 hr. Of the 639 females used, 326 females were tested on human blood, 148 on chicken blood and 165 on duck blood. The results are tabulated in Table 3 and the trends of the feeding and lack of feeding are represented in Graph 1. Evidently engorgement increased as the proportion of sucrose in the blood-sucrose mixture increased. Some flies fed partially on whole human blood but took a full meal only when sucrose comprised 30% of the blood-sucrose mixture. With the duck blood, feeding commenced only when some sucrose was added to the blood, and with the chicken blood, more than 20% sucrose was needed in the mixture to stimulate any feeding. The number of flies fully engorged on the 1:1 mixture of either avian blood was much less than with the human blood-sucrose mixture. This is consistent with the observation in nature that mammalophilic species seldom feed on ducks and rarely can be induced to feed on chickens, even when held near the skin in screened tubes.

When five females, partly or fully fed on each human, duck and chicken blood-sucrose mixture, were dissected 5 hr later to check whether the meal was in the crop or in the midgut, most of the meals were found in the crops, except for flies fed on whole human blood in which the meal was found only in the midgut.

(b) Distribution of Blood-Sucrose Mixtures to the Crop or the Midgut of S. venustum Females

Although most of blood-sucrose mixtures were found in the crops in the above experiments, this provided an insufficient trend of distribution of the mixture in the female alimentary tract, presumably because saturated sucrose solutions were used. Therefore, the following tests were performed to determine specifically what concentration of sucrose in blood was responsible for sending the mixture to the crop or to the midgut in the flies. S. venustum females, 72 hr after emergence from pupae in the laboratory, were grouped in the cardboard cylinders. Citrated human blood was serially diluted with 1 M sucrose solution and the prewarmed blood-sucrose mixtures, soaked in cotton, were placed on the top screen of the cylinders. One hour later the flies were dissected and the distribution of the mixture was examined (Table 4). Although the blood-sucrose mixtures were found in both crop and midgut in six of the seven mixtures tested, the amount of mixture in the crop varied considerably. With solutions of 0.5 M sucrose in blood, all of the 10 females had most of the mixture in the crop, with only traces in the midgut. With 0.4 M sucrose, 15 of 18 females had most of the mixture in the crops and 3 had much of the mixture in the midgut with a little in the crop. With 0.3 M sucrose 7 of 11 females had more of the mixture in the midgut and 4 had more in the crop. In 0.2, 0.1 and 0.05 M sucrose, only a small amount of the mixture was ingested by the females and the mixtures were equally distributed in both crop and midgut, and no females had the mixture in the crop alone. Only 4 of 50 females offered 0.01 - 0.02 M sucrose in the blood took traces of the mixture and these small meals were found only in the midgut.

Although no females of S. venustum provided with 70% human plasma in 1 M sucrose solution were attracted to this mixture, more than half (195 females) offered 70% human erythrocytes in the sucrose solution ingested the mixture.

An attempt was made to determine the volume of crop contents of S. venustum females after feeding on various mixtures of human erythrocytes and sucrose solution. Since the mixture in the crop was stored in a liquid state, it was also possible to determine the number of blood cells in the crop content. The determination of the volume of and the number of the cells in the crop could be accomplished at the same time by using a blood cell counting chamber (Neubauer ruled chamber, with 1/10 mm depth). When the abdominal appearance of females suggested that they were fully engorged, the volume of the crop ranged from 1.2 to 1.35 µl (Table 5). Whether the females were provided with a greater or lesser number of erythrocytes in a mixture, the number of blood cells ingested into the crop differed little. Thus females which ingested mixtures of 1 M sucrose and 555,000 blood cells per µl, revealed 45,000 to 54,000 cells per µl crop content, while females fed on mixtures with 58,000 cells per µl showed 34,000 to 43,000 cells per µl crop content.

(c) Sensory Receptors in the Buccal Cavity of Female Simuliids

The feeding experiments with mixtures of blood components and sucrose solution suggested that there might be some types of sensilla necessary to detect both sugars and blood, and direct them to either the crop or midgut.

Serial sections and mounted dissections of the head and thorax were prepared for the following species: S. decorum, S. quebecense,

S. venustum, S. vittatum; serial sections only from: P. decemarticulatum, S. aureum, S. croxtoni, S. rugglesi and mounted dissections from: P. fuscum, P. gibsoni, C. invenusta, C. mutata, S. excisum, S. latipes, S. luggeri and S. verecundum.

Examination of the preparations from 16 species of black-flies revealed no sense organs on the hypopharynx, or in the pharyngeal pump and oesophagus. However, on the ventral (epipharyngeal) surface of labrum and in the cibarial pump, several groups of spines were observed in all of the species examined. Careful observations of serial sections and whole mounts of the labrum and cibarial pump revealed that at least four groups of sensilla occurred, one group in the cibarial pump and the other three on the labrum. All the spines pointed slightly toward the back of the buccal cavity. The location and arrangement of the sensilla are shown in Fig. 2.

On the ventral surface of the labrum at least three groups of sensory spines occurred: a terminal, a lateral and a medial group. The terminal group, situated on the terminal palatum of the labrum, usually comprised four spines but often five or six in certain species. Some specimens of P. fuscum, P. gibsoni, S. quebecense and S. vittatum of the 16 species examined showed five to six spines in the terminal group, although four spines were seen more often in these species. The lateral group was located on the ventro-lateral palatum of the labrum along each side from the proximal edge of the terminal palatum to almost the proximal end of the labrum. The number and arrangement of the lateral sensory spines varied according to specimens and species. P. fuscum and C. invenusta had 60 to 80 spines in the lateral group and the other species, such as P. gibsoni, C. mutata, S. decorum, S. excisum, S. latipes, S. luggeri, S. quebecense, S. venustum, S. verecundum and S. vittatum,

had 30 to 50 spines. The medial group in the middle of the medial palatum of the labrum, usually comprised two sensory spines but some females of P. gibsoni and C. mutata revealed one spine or none.

The sensilla of the medial group and a pair of the terminal group had slightly curved and heavy spines (length 5-6 μ) with membraneous bases (diameter of the base 7-8 μ), whereas the lateral group had slender and short spines with heavily sclerotized rings around membraneous bases (diameter of the base 3-4 μ)(Figs. 2, 10, 11). The spines were innervated by very fine dendrites leading from a small group of cells which lay underneath the sensory spines, and the group of cells were connected to the labral nerve (Figs. 2, 14).

In the middle of the cibarial pump, one sensillum was located on each side of the latero-ventral surface. Each sensillum consisted of two slightly curved and heavy spines (length about 8 μ) which arose from a membraneous base (diameter of the base 9-10 μ)(Figs. 12,13). Unlike the labral sensilla, the sensilla in the cibarial pump were of the same number (i.e. two) and form in all of the 16 species examined. Each spine was innervated by fine dendrites originating from cells closely associated with them.

At the junction of cibarial pump and pharyngeal pump, a number of strong spines or papillae were observed in several species of simuliids examined (Figs. 15, 16, 17). S. vittatum females had numerous strong spines, while those of S. decorum, S. luggeri, S. venustum, and S. verecundum, have a few minute papillae. The other species examined, such as P. fuscum, P. gibsoni, C. invenusta, C. mutata, S. excisum, S. latipes and S. quebeconse, showed no such spines or papillae. No innervation of these spines or papillae was observed.

(d) Agglutinin and Anticoagulant Factors in the Salivary Glands and Crop of Female Simuliids

No proteinase enzymes or carbohydrase activity was detected in the salivary glands of the adult black-fly females (see Results, sections IV and V, under Digestive Enzymes). However, agglutination and anticoagulant factors were present in these glands.

(i) Agglutinin activity: The addition of human erythrocytes to the salivary gland extract resulted in a marked agglutination or clumping of the cells. The action of agglutinins from three different species of black-flies, S. decorum, S. venustum and S. vittatum, was tested against blood cells from four different animals: human, cow, duck, and chicken (Table 6). In S. decorum females, no agglutination occurred with glands from flies less than 2 hr old, perhaps because the agglutinin had not yet developed. However, when the salivary gland extracts from females 12-24 hr old were mixed with human blood cells, agglutination occurred usually within 10 min. The appearance of agglutinin activity was related to the size of the salivary glands (Table 2). Positive agglutinin activity was shown when 11 of 15 salivary gland extracts of S. venustum females were mixed with human blood cells, 5 of 7 extracts with duck blood cells and all of 7 extracts with chicken blood cells, but not when any of 10 extracts were mixed with cow blood cells. On the other hand, with the gland extract from females of S. decorum all of 15 extracts with human blood cells and 12 of 13 extracts with duck blood cells showed positive results, but no agglutination was observed with cow and chicken blood cells. When 5-8 day old S. vittatum females, which had fully grown ovaries, were tested, they gave strong agglutination in 18 of 20 tests on human and duck blood cells.

After heating the salivary gland extract of S. venustum females in saline at 50°C for 10 min, it showed no agglutinin activity. However, when intact females were heated at the same temperature for 1 hr and the glands subsequently removed and tested, agglutination of blood cells occurred within 6 to 10 min.

Agglutinin activity of the salivary reservoirs, as well as distal and proximal arms of 5 females of S. venustum tested separately revealed strong agglutination. In one case, one salivary reservoir in 5 µl was diluted 1 : 2, 1:4 and 1:8, and agglutination of the blood cells occurred in the 1:4 dilution. A pair of salivary glands from a S. venustum female was extracted in 10 µl saline and diluted serially 1:2, 1:4, 1:8 and 1:16. Strong agglutination was observed in the 1:4 dilution and weak activity in the 1:8 dilution.

(ii) Anticoagulant activity: The standard clotting time of recalcified oxalated cow plasma was found by our method to be between 3 and 5 min. The clotting time was delayed, however, when salivary gland extract was added. The delay in coagulation of oxalated plasma with the addition of 5 to 10 µl of gland extract varied from 6 - 40 min, in different species and also in different individual females (Table 7). Thus addition of the extracts from S. decorum delayed the clotting time by 6 - 14 min, from S. vittatum by 6 - 15 min, but from S. venustum by 8 - 40 min. Values within the three species varied widely, and differences in means were not statistically significant.

No anticoagulant activity was demonstrated with salivary gland extracts which had been heated at 100°C for 30 min.

(iii) Agglutinin and Anticoagulant Activity of Crop Contents:

Crop contents of S. venustum females were tested for both agglutinin and anticoagulant activities. Since it has been proved in previous experiments that an appropriate dilution of a blood-sucrose mixture goes to the crop, an attempt was made to test the agglutinin factor in vivo. Females were given human blood cells mixed with a sucrose solution, and the crops containing the mixture were dissected to test for agglutinin activity. The same mixture of blood cells and sucrose was tested with extracts of salivary glands as standards with known agglutinin activity. No agglutination of the erythrocytes in the crop was observed in over 50 flies studied, while standards showed strong positive results.

Five crops from individuals of S. venustum which were caught by sweeping around a human host in Algonquin Park on June 1, 1967, were tested for anticoagulant activity with negative results.

III The Peritrophic Membrane and Blood Digestion in Adult Simuliids

The main questions in this aspect of the study were whether a peritrophic membrane (PM) forms in the midgut of a black-fly before and after a blood meal, how the PM disappears after the blood is digested, and whether a blood-sucrose mixture which goes to the crop induces a PM in the midgut. These points were investigated exclusively by histological and morphological observations from sections and dissections of the flies.

The peritrophic membrane was found to be composed of chitin, as membranes dissected from females 12-24 hr after a blood meal gave a positive chitosan test (see Materials and Methods).

(a) Peritrophic Membrane Before Blood Meal

Unfed black-fly adults were examined for the presence of a PM, before studies of the PM in blood-fed flies were made. Both sexes of S. decorum, which had emerged from pupae in the laboratory and had been provided only with sugar and water for two days, were processed for histological studies. Serial sections (both longitudinal and transverse) from five males revealed that, in two of them, a thread-like material was seen in the anterior tubular part of the midgut near the proventriculus (Fig. 18). This material, "the old gut content", extended to the back end of the tubular midgut but was not seen in the posterior midgut. In sections of one of the 10 females sectioned, similar material was observed in the posterior midgut but with a different shape (Figs. 19, 20). The material, "the old gut content", stained with haematoxylin, and stretched as a thread from the anterior to posterior midgut where the material became pear-shaped. The pear-shaped material contained fine granular and gritty substances. Such materials with slightly different shape were frequently found in the midgut of blood-fed females, especially within a few hours after a blood meal or at the end of digestion.

(b) Formation of Peritrophic Membrane and Process of Digestion after a Blood Meal

In a search for possible differences in the processes of peritrophic membrane formation, blood digestion, and disappearance of the PM in the midgut of the black-fly, females of five ornithophilic and one mammalophilic species of black-fly were examined at various intervals after feeding on different hosts.

Females of S. venustum, a mammalophilic species, which ingested

blood of human, deer, moose or duck, formed generally a thinner PM than ornithophilic species, such as P. decemarticulatum, S. aureum, S. croxtoni and S. quebecense which fed on chicken blood, and S. rugglesi which fed on duck blood. Nevertheless, serial sections from 350 females representing the six species revealed that the overall process differed little regardless of the black-fly species or the type of host's blood ingested, although details in the pattern of the process varied even within one species. Therefore, the general changes in the appearance of the PM and the digesting blood will be described, instead of describing the pattern in each species.

Sections made immediately after female black-flies had taken a full blood meal showed the abdomen to be round or oval. In many females blood was found in the tubular midgut of the thorax, but unlike the posterior midgut, the tubular part did not distend. In a few flies the blood remained in the tubular part for 6-12 hr until the blood volume in the expanded posterior midgut decreased.

At first the blood in the midgut was in direct contact with the striated border of the midgut wall (Fig. 21), and apparently no PM substance was secreted. In most of the specimens examined, an "old gut content", extending from the back end of the tubular midgut to the pyloric region of the posterior midgut, was frequently found, surrounded by freshly ingested blood (Figs. 22, 47, 48, 52). This was usually gripped by the pyloric sphincter and sometimes projected into the hindgut (Fig. 22).

In sections made from females 1 hr after a meal, the blood was now completely enclosed by a smooth and viscous substance. The thickness of the viscous sheet varied markedly among specimens, ranging from 3 μ to 60 μ. Even in the same females, the sheet was thicker and seemed more viscous

in the pyloric region than anteriorly. The blood with some gritty particles in the anterior midgut was also enveloped by a thin membrane (Figs. 41, 42), and this material ("old gut content") was slowly moved down to the posterior midgut. When females were dissected 30 min after a blood meal, the intact PM in the tubular midgut was easily separated from the gut wall but the PM in the expanding posterior midgut could not be separated intact at this time. It appeared to be a gelatinous and transparent mass which adhered to the blood mass and to the gut wall. At this time the blood mass showed no sign of digestion.

In most females sectioned 3 hr after blood feeding, the viscous substance (peritrophic membrane) began to stabilize from the posterior to the anterior region of the midgut, and to solidify first at the outer surface of the membrane nearest the gut wall. In a few females a series of laminated layers could be observed in the region of postero-ventral midgut (Fig. 23). Occasionally the PM was detached from the gut wall at this time, although the detachment occurred later in most specimens. In one female of S. aureum which had a large blood meal, the membrane in the region of the pyloric sphincter showed four layers, alternately laminated and unlaminated, from the outermost one near the gut wall toward the interior ones (Fig. 24). Only one of 25 females sectioned at 3 hr showed a slight indication of digestion, as some blood cells had lost their structure along the periphery of blood mass in pyloric region.

In females sectioned 6-12 hr after a blood meal, the viscous membraneous substance began to harden and wrinkle on the periphery, the rest consisting of as many as seven laminae (Fig. 25). At this time the entire PM was easily detachable from the gut wall, but still firmly attached to the blood mass (Fig. 26, 27). In general the membrane at the posterior end of the

blood mass was thicker and firmer than that of the dorsal or ventral region. No marked digestion of blood was noticed, although a digested product was observed between the membrane and the gut wall at the region of the posterior end of the midgut.

A double membrane was occasionally observed in one or two females of all species examined. Some females showed two membranes distinctly, an inner one being completely surrounded by a second blood meal which in turn was enclosed by an outer membrane (Fig. 27). On the other hand, some females showed a partly divided double membrane, the first and second blood meal being only partly separated by the inner membrane which was connected to the outer one. Another unusual feature of the membrane was observed in f[illegible] females of S. quebecense. In these cases, it appeared as if the primary membrane had ruptured 6 or more hours after feeding and that the flies had been killed before a new membrane had formed around the escaped blood. In two of these females, this break in the membrane occurred at the anterior end of the blood mass, while in the other two, it occurred at the posterior end near the pyloric sphincter (Figs. 26, 28, 29).

In females sectioned 24 -48 hr following a blood meal, the whole PM in some females had solidified to an almost compact membrane and had begun to shrink, perhaps because of the decrease in blood volume (Fig. 30), and in some others the membrane seemed to have relaxed or begun disintegrating (Fig. 31). A small amount of the amorphous mass of altered blood pigment, which appeared black in the sections stained with haematoxylin and eosin, was seen in the posterior end of the blood mass within the peritrophic membrane which was disintegrating. The total blood volume in the gut was reduced at this time to about two-thirds of the original volume.

In females sectioned 72 - 96 hr after a blood meal, digestion had now markedly progressed, most evident at the periphery of the blood mass, especially at the posterior end with progressively less digestion anteriorly. While digestion continued, the peritrophic membrane was incorporated with the altered blood pigment and gradually broke down (Figs. 32, 33, 34). In most females the membrane became invisible in the region occupied by the black pigments.

In females sectioned 120-168 hr after a blood meal, the much reduced blood mass in the midgut was pushed anteriorly, because most of the abdominal space was occupied by the maturing eggs, and the blood mass was enclosed by the altered blood pigments, with undigested blood still remaining in the center. In a few females the membrane was intact in an anterior region where the membrane enclosed the undigested blood (Fig. 35).

In one female of S. rugglesi and one of P. decemarticulatum, the ingested blood was completely digested within 120 hr. However, 18 females of the above species and of S. aureum showed a small amount of undigested blood until 160 - 183 hr after a blood meal (Figs. 36, 37). At the final stage of blood digestion, the disintegrated hind part of the peritrophic membrane was discharged with the waste products into the hindgut (Figs. 38, 39, 40), while the membrane at the front end of the expanded midgut was still disintegrating.

In some females of S. aureum, 3 hr after feeding, the blood residue in the anterior tubular midgut was completely enclosed in a gelatinous substance which moved posteriorly to form a cap at the anterior end of the large blood mass in the posterior midgut (Fig. 43). When sections prepared 48 hr after the meal were examined, this viscous substance ("old gut content"), containing some gritty particles, formed a larger and solidified cap (Fig. 44). In several females of S. aureum and S. quebecense which were killed 144-168 hr

after a blood meal, many gritty particles or other organic materials, completely enclosed by a membrane, were observed in posterior region of the tubular midgut (Figs. 45, 46). In sections of a few flies in which most of blood mass in the midgut was digested and waste products excreted, this substance enclosed by a membrane, originally from the anterior midgut, was found in the posterior midgut. The midgut of six females sectioned more than 200 hr after a meal showed no blood, but two contained material similar to the so-called "old gut content" in the posterior midgut.

A few females of P. decemarticulatum and S. ruggloi which were heavily parasitized with microsporidia in the ovaries showed a fair amount of undigested blood even after 200 hr. In these flies, whole ovaries were occupied with the microsporidia and when a few eggs were found in the parasitized ovaries, they were only partly developed.

(c) Peritrophic Membrane after a Meal of Blood-Sucrose Mixture

It has been shown previously that an appropriate mixture of blood and sucrose solution goes to the crop of the black-fly and is slowly dispatched to the midgut. An experiment was designed to investigate whether such a blood-sucrose mixture would induce peritrophic membrane formation in the midgut of the black-fly females.

Groups of S. venustum and S. vittatum females, which emerged from pupae in the laboratory, were given either 70% whole human blood or 70% cow blood cells in a 1M sucrose solution. Most of these mixtures went to the crops in both species, although in several females more of the mixture was found in the midgut than in the crop. To check whether the mixture in the crop was actually dispatched to the midgut, three females of S. venustum, which were provided with cow blood cells, were sectioned 3 - 6 hr later. In

all of three flies, the mixture was observed in the crop, crop duct and proventricular region (Figs. 49, 50). Since these flies were killed more than 3 hr after ingesting the mixture, this clearly indicates that the mixture in the crop is sent to the midgut.

When the mixtures, whether a trace or large quantity, went directly to the midgut, a thin membrane surrounding the mixture in the posterior midgut could be observed in the females sectioned 5 - 12 hr following feeding (Fig. 51). In one female of S. venustum which fed on human blood-sucrose mixture twice at about a 30 min interval, the first meal was completely enclosed by a delicate membrane and the second meal partly by another thin membrane at its posterior margin. However, no peritrophic membrane could be observed in the crops which had the same mixture.

In 10 females of S. venustum and 15 females of S. vittatum sectioned 24 and 48 hr after feeding on human blood-sucrose mixture, all of the females showed the mixture both in the midgut and crop. However, no membrane could be observed in the midgut of the females, except that one female of S. vittatum showed an "old gut content" which was gripped by the pyloric sphincter (Fig. 52).

IV Proteinases in Adult Simuliids

(1) Trypsin-like Enzyme

(a) Standard Curve

The standard curve (Graph 2) was used to convert optical density of the unknown solutions into trypsin equivalents.

(b) Occurrence of Trypsin Activity in Various Tissues of Female Simuliids

Since preliminary experiments, using homogenates of whole black-fly bodies as an enzyme source, demonstrated a detectable amount of trypsin

activity in sugar-fed flies, an assay was made to determine whether the trypsin activity was limited to the midgut or was present in other tissues.

Forty sugar-fed females of S. venustum, an anautogenous species, were dissected 6-7 days after their emergence from pupae and various tissues assayed for the enzyme activity (Table 8). Little or no trypsin activity was detected in salivary glands, Malpighian tubules or carcasses. The weak trypsin activity found in residual fluid after dissection was believed to be derived from midgut contents which may have been squeezed out while separating other tissues in the dissecting fluid. The midgut always showed the highest activity, suggesting that most, if not all, of the trypsin was located in the midgut. When two suspensions of pooled hindguts (15 hindguts/ml) were tested, one group showed no activity, while the other showed a trace (less than 0.1 µg trypsin/ml), presumably contamination from the midgut contents.

Fifty females of S. venustum, which had fed on a mixture of human blood and sucrose, were dissected at different times after the meal and trypsin activity was determined in various tissues (Table 9). The carcasses including salivary glands, Malpighian tubules, and also crops which were filled with the blood-sucrose mixture, showed only an occasional trace of enzyme activity, the trace again believed to be a contamination from the midgut contents. Trypsin activity in the midgut, however, had sharply increased after the meal, compared with the activity in the sugar-fed females (Table 8). Because trypsin activity had been noted in the residual fluid of sugar-fed S. venustum females (Table 8), more careful dissections were made of 30 females of S. vittatum, a mammalophilic species autogenous for the first gonotropic cycle, and various tissues were tested for enzyme activity (Table 10). In this species trypsin activity in the residual fluid was negligible,

when compared with activity in the midguts. The carcasses, which included ovaries with fully grown eggs, showed no activity.

The total trypsin activity obtained in the midgut and other tissues tested separately agreed well with that found in whole flies (Tables 8, 10). In S. venustum females fed on blood and sucrose mixture (Table 9), there was little difference in trypsin activity between the midgut and the whole fly.

The foregoing experiments indicated that most of the trypsin activity was located in the midgut and that use of homogenates of whole fly interfered little with determining trypsin activity in the midguts. Therefore, the remaining experiments, on the analysis of trypsin activity in the black-fly, were performed with homogenates of whole flies.

(c) The Effects of Incubation Time, pH, Substrate and Homogenate Concentrations upon Trypsin Activity

To determine the relation between incubation time and enzyme activity, homogenates of blood-fed females of two different anautogenous black-fly species, P. decemarticulatum and S. rugglesi, were used (Graph 3). With a homogenate of P. decemarticulatum females about four days after blood-feeding on a bantam chicken, the hydrolysis of the substrate increased rapidly for the first hour of incubation and then remained more or less constant. This suggested that there was insufficient substrate left after 60 min of incubation to be hydrolysed by the highly active homogenate. This was proved by results with a 10-fold dilution of the homogenate; the rate of hydrolysis was constant for at least the first 2 hr. A homogenate of S. rugglesi females (6 - 18 hr after blood-feeding on a Pekin duck) showed linearity during the 2-hr incubation period.

A ten-fold increase in the concentration of the homogenate of P.

decemarticulatum females resulted in a similar increase in trypsin activity (e.g., Graph 3 at 30 min) suggesting a linear relation over this range of concentration.

The pH optima for trypsin activity in females of S. venustum fed on a blood-sucrose mixture, and of S. rugglesi fed on duck blood were determined by preparing homogenates of whole flies, ground up in cold distilled water which had been neutralized with diluted NaOH and/or HCl. The homogenate was then adjusted with buffer solutions to a final concentration of about 4 females/ml for S. venustum or of about 5 females/ml for S. rugglesi. In the three buffers[15] used, the trypsin in the two species showed similar activity, being active between pH 8 and 8.5 with a maximum activity at pH 8.4 (Graph 4).

The effect of substrate concentration upon the trypsin activity of S. rugglesi and S. venustum homogenates is shown in Graph 5. With the data obtained from substrate concentrations below the point at which the enzyme is saturated, the values of the Michaelis-Menton constant (Km) were calculated, applying the Lineweaver-Burk double reciprocal plot of the activity and substrate concentration(Graph 6). The Km for the S. venustum homogenates was 2.4×10^{-3}M and for the S. rugglesi homogenates 3.1×10^{-3}M.

(d) Trypsin Activity in Sugar-Fed Flies at Intervals After Emergence from Pupae

Adults of P. decemarticulatum, C. dacotensis and S. venustum, supplied only with dry sucrose and water, were tested for trypsin activity at intervals after the flies had emerged from pupae. Each black-fly species tested showed a more or less constant level of trypsin activity 1 - 24 hr after emergence from pupae (Graph 7). It was obvious that sucrose stimulated no

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15. M/5 Tris aminomethane/HCl buffer
Boric acid/borax buffer
Citric acid/HPO_4 buffer

trypsin activity since these flies began feeding on sugar within few hours after emergence.

(e) Comparison of Trypsin Activity in Male and Female Simuliids

Since it is only the female black-fly which is capable of piercing and sucking blood from vertebrates and which must digest this blood to supply nutrients for oogenesis, it was of interest to compare the trypsin activity in the two sexes.

Before females of P. decemarticulatum, P. fuscum and S. venustum took blood, their enzyme activity was practically identical with that of the males of these species, and that of males and females of C. dacotensis, a non-blood feeder, were also similar (Table 11).

(f) Trypsin Activity in Female Simuliids at Intervals after a Blood Meal

The subsequent experiments were undertaken in order to determine how much the trypsin activity increased in black-flies after a blood meal. S. rugglesi females which fed on duck blood and P. decemarticulatum females which fed on chicken blood were analysed for trypsin activity at different times following the blood meal. The pattern of increasing trypsin activity in the two species after a blood meal differed (Table 12 and Graph 8). In S. rugglesi there was a gradual increase up to 18 hr followed by levelling off, whereas in P. decemarticulatum there was a sharp increase before 5 hr and then the activity remained more or less constant for at least 24 hr. In the females of S. venustum which fed on human blood, trypsin activity 3 hr thereafter was more than doubled when compared with the activity immediately after a blood meal. Trypsin activity in 10 females of S. quebecense, 1 hr after a chicken blood meal, was equivalent to 7.4 µg trypsin per ml homogenate, but increased to 16.9 µg per ml when females of the same species were analysed 72 hr later. The enzyme activity differed in females of P.

decemarticulatum and S. quebecense both of which fed on the same host and were analysed for trypsin activity 72 hr later, the latter being twice that of the former (Table 12).

(g) Trypsin Activity in Female Simuliids at Intervals After a Meal of Blood-Sucrose Mixture

As shown in previous experiments, unlike whole blood which directly went to the black-fly midgut, most of the blood-sucrose mixture was initially dispatched to the crop, but as this mixture must eventually pass slowly into the midgut for digestion, the following questions arose: a) Is the trypsin present in the midgut before a blood meal sufficient to digest all the blood coming to the midgut from the crop or is extra trypsin required? b) If the trypsin activity increases following the feeding of the mixture, what is the pattern of the increase in trypsin activity? c) How does the temperature affect the trypsin activity in females fed on the mixture? d) If male black-flies feed on the mixture, does their trypsin activity increase after feeding?

A mixture of citrated human blood and 1 M sucrose solution (1:1) was given to about one-week old male and female simuliids which were previously starved for 24 hr. The partially and fully engorged females were kept at two temperatures, one group at 30±1°C.

Determinations of trypsin activity in the flies were made at various times after they fed on the mixture (Graph 9). The data on trypsin activity, obtained in females kept at both temperatures, were analysed for second degree polynomial curve fitting. The polynomial

expressions were obtained from an IBM computer and evaluated to obtain the theoretical values of Y for the corresponding values on the X axis and these values were plotted against each other. At 15°C, trypsin activity in the females was apparently less active than in the females at 30°C. However, they showed similar patterns of enzyme activity during the test period. It was notable that the increased trypsin activity could be maintained for more than 8 days after the females fed on blood-sucrose mixture. Although the trypsin activity in the females at 15°C was less than at 30°C, it was still higher than the activity in sugar-fed females, suggesting that enzyme activity was stimulated by the blood-scurose mixture even at the low temperature. On the contrary the trypsin activity in males at 15°C was not increased by the blood-sucrose mixture; in fact the activity was somewhat less than that in unfed males (see Table 11).

(h) Comparison of Trypsin Activity in Female Simuliids After a Meal of Whole Blood or of Erythrocytes Suspended in Sucrose Solution

Since the blood-sucrose mixture stimulated increased trypsin activity in S. venustum females, further experiments were conducted to determine what fraction of the blood was essential for stimulating enzyme production in the midgut of the females.

One batch of S. venustum females was fed on duck erythrocytes which were thrice washed in isotonic saline and then suspended in 1 M sucrose solution to make an 80% suspension, while another batch was fed on 80% duck whole blood diluted in the same sucrose solution. A

plasma-sucrose mixture (4 : 1) was tried but the flies were reluctant to feed on this mixture. Trypsin activity in the females was determined at different times after they had partially or fully fed on the blood-sucrose or blood cell-sucrose mixture (Table 13). Similar experiments were conducted with oxalated cow blood and erythrocytes, and the enzyme activity was determined (Table 14). Trypsin activity in females fed on a mixture of duck erythrocytes and sucrose was slightly lower than that in females fed on whole duck blood in sucrose solution during the test period, while trypsin activity in the females fed on the cow whole blood-sucrose mixture or on erythrocytes suspended in sucrose solution differed little. In either case, the erythrocytes suspended in sucrose solution apparently stimulated enzyme secretion almost as much as whole blood diluted in the sucrose solution.

(2) Pepsin-like Enzyme

For the enzyme source, a homogenate consisting of 12 flies per ml glycine-HCl buffer (pH 2.5) were prepared from unfed adult males and females of P. fuscum, a mammalophilic black-fly species autogenous for the first gonotrophic cycle. Incubated mixture with the homogenate and substrate were centrifuged after addition of 10% TCA and treated with 0.5 N sodium hydroxide to intensify the colour of the incubation mixture.

Determination of optical density from experimental tubes of male and female homogenates differed little, when compared with those obtained from tubes of boiled homogenates or with residual colour of the substrate alone. To insure the method, crystallized pepsin was serially diluted in the glycine-HCl buffer to consist of 50, 10, 5, 1 µg/ml pepsin in each dilution. Each concentration of pepsin was added to a homogenate of females and analysed for pepsin activity (Table 15). Optical density increased in almost direct proportion to the amount of added pepsin in the experimental tubes when compared with tubes of boiled homogenate, indicating that the method was adequate to detect pepsin activity in these concentrations. It is thus believed that the black-fly homogenates assayed contained no pepsin-like enzyme.

V Carbohydrases in Adult Simuliids and Culicids

(1) Amylase Activity in Adult Simuliids and Culicids

(a) Amylase Activity in Homogenates of Whole Black-Flies with or without Bacteriostat

Although a preliminary test with homogenates of sugar-fed S. venustum females indicated considerable starch-hydrolysing activity during a 60-min incubation period, it was considered that part or all of the activity may have resulted from contamination by microorganisms. Therefore, tests were made to find whether the enzyme activity was derived from the fly itself or from bacteria or other microorganisms present in or on the fly. The reaction mixtures, including homogenate, were incubated with and without toluene as a bacteriostat and the results were compared (Table 16). The optical densities of toluene-free or toluene-containing incubates were essentially identical. It is believed that starch-hydrolysing activity was derived from the fly itself rather than from microorganisms. This was further substantiated by bacteriological and microscopical tests on sediments of toluene-free, 60-min incubation mixtures. No microorganisms were found in several microscopical examinations and only two small bacterial colonies developed in nutrient agar when six sediments were incubated for 48 hr.

The above experiment was also designed to determine whether there was a difference in the starch-hydrolysing activity of the two sexes. As seen in the Table 16, the optical density of homogenates of sugar-fed S. venustum males (homogenate 3) was lower than that of homogenate of females (homogenate 2) at the same age. The lower enzyme activity of the male compared with the female was also observed when two similar homogenates

were studied for the effect of hydrogen ion concentration (Graph 11).

(b) Occurrence of Amylase Activity in Various Tissues of Adult Simuliids and Culicids

In adult haematophagous Diptera which have proteins the main component of their diet, it is unlikely that amylase would be actively secreted in the midgut for digestion of the protein diet. Therefore, it seemed reasonable to assume that the starch-hydrolysing activity demonstrated in the homogenates of whole flies, may have been derived from a tissue or tissues other than the midgut. Various tissues of sugar-fed females of S. venustum and S. vittatum, as well as whole flies, were therefore assayed for amylase activity. Various tissues of two mosquito species, A. aegypti and C. p. pipiens, were also examined for amylase activity in tissues other than the midgut. Organs, such as salivary glands, crops and ovaries, showed no amylase activity in any of the four species. Most of the enzyme activity in each species appeared to be in the residual fluid and in washings from the carcasses which were considered as additional residual fluid (Table 17). The actual enzyme activity in the midguts of four species, and in carcasses of S. vittatum, would have been even lower than is shown in the Table 17 if they had been washed before testing for amylase activity. The residual fluid consists of mainly of haemolymph with a small amount of fat body and other tissue fragments in the dissecting fluid. That there was very weak or negligible amylase activity in the midgut of four species of bloodsucking insects proves the previous assumption that amylase is not active in the midgut of these haematophagous insects.

The starch-hydrolysing activity in the whole-body homogenates of the S. venustum was higher than that of S. vittatum. It is unknown

whether this is related to the fact that S. venustum females are anautogenous whereas S. vittatum females are autogenous for the first cycle of eggs. It is of interest that hibernating C. pipiens females possess higher amylase activity than non-hibernating A. aegypti females.

(c) The Effects of Incubation Time, pH, and Substrate Concentration upon Amylase from Black-Flies

The rate of starch-hydrolysis by homogenates of sugar-fed females of both P. fuscum and S. venustum was constant at least for the first 1.5 - 2 hr of incubation when a 1% starch solution was used (Graph 10).

The optimum hydrogen ion concentration for amylase activity was determined by preparing homogenates of sugar-fed males and females of S. venustum (Graph 11). With flies of either sex, considerable enzyme activity occurred between pH 6 and 7, with maximum activity at pH 6.5. The presence of toluene did not alter the pH optimum.

The effect of substrate concentration upon amylase activity is shown in Graph 12. Maximum activity of the amylase in females of S. venustum was obtained when the concentration of starch in the reaction mixture was about 2.5 mg/ml. With the data obtained from the substrate concentrations, the value of the Michealis-Menton constant (Km) was calculated. The reciprocals of the activity and substrate concentrations were fitted with a straight line by applying the Lineweaver-Burk double reciprocal plot. The Km for S. venustum homogenates was 6.5×10^{-1} mg/ml (Graph 13).

(d) Amylase Activity in Female Simuliids at Intervals after Emergence from Pupae

To determine whether amylase activity in a female black-fly remains constant or gradually increases after adult emergence, homogenates of sugar-fed S. venustum females were tested at various intervals during the first

24 hr of adult life. The enzyme activity was similar from 1 - 6 hr, but at 9 hr it almost doubled and then remained nearly constant for at least 24 hr (Table 18). All flies had fed on sucrose which was provided shortly after emergence, but it is uncertain whether sugar-feeding increased this enzyme activity in the fly after 6 hr. In sugar-fed A. aegypti females, however, no increase in amylase activity was seen during the 72-hr test period (Table 19).

(e) Amylase activity in Female Simuliids and Culicids after Feeding on Blood

Although a weak amylase activity was observed in the midguts of sugar-fed black-flies and mosquitoes (Table 17), it is still uncertain whether blood feeding will stimulate an increased amylase activity in the midguts of these bloodsucking insects. Because there were few blood-fed black-fly specimens available for this experiment, A. aegypti mosquitoes were substituted.

One batch of females of A. aegypti was allowed to feed on a human host and homogenates were prepared from engorged mosquitoes at various times after a meal. In addition, two other batches of female mosquitoes were continuously provided with 10% boiled starch[16] or with 10% sucrose solution for 29 hr, after which homogenates were prepared in the same way as from blood-fed mosquitoes. Analysis of starch-hydrolysing activity in the homogenates are shown in Table 19. Mosquitoes provided with sucrose or starch for over 24 hr showed an amylase activity similar to that of unfed mosquitoes, indicating that feeding on starch or sucrose did not increase

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16. Since the females were reluctant to ingest the starch solution alone, 10% sucrose solution was added to the starch solution.

enzyme activity. However, in mosquitoes before and after blood feeding, there was a great increase in the amylase activity immediately after the blood meal. This activity, however, gradually decreased while the blood meal was being digested, which was contrary to the trypsin activity in the midgut of blood-fed flies.

Under the assumption that the amylase activity immediately after a blood meal may have been derived from host blood rather than from the mosquito itself, the enzyme activity in the host's blood was compared with that in homogenates of freshly blood-fed mosquitoes. Females of A. aegypti were allowed to feed fully on a human host, and immediately after engorging, the mosquitoes were ground up and homogenates were prepared at a concentration of 10 mosquitoes per ml distilled water. At the same time, blood was drawn from the human host which had been exposed to the mosquitoes. Considering the volume of blood in a fully engorged A. aegypti female to be 2.6 µl (Jeffery, 1956)) 26 µl of the drawn blood, equivalent to the blood volume in 10 engorged mosquitoes, were diluted in 1 ml distilled water. These preparations were analysed for amylase activity. The enzyme activity in the blood-engorged mosquito and in the host blood was almost identical (Table 20). This indicates that the amylase activity in blood-fed A. aegypti females resulted mainly from the ingested blood rather than from midgut secretion.

With limited specimens a preliminary test was made to find whether blood feeding in S. venustum females stimulated any increase of amylase activity in the midgut. Homogenates of S. venustum females which had fed on duck blood 1 hr and 48 hr previously were tested for amylase activity. The optical density obtained from the females 1 hr after the blood meal was 0.73 per 5 flies in 1 ml homogenate. However, 48 hr after a blood

meal, the optical density had decreased to 0.45 per 5 flies, which differed little from that of sugar-fed flies.

(2) Invertase Activity in Adult Simuliids

(a) Standard Curve

A standard curve was drawn from the optical density readings against standard glucose concentration (Graph 14). The curve was used to convert optical density obtained from unknown materials into a glucose concentration equivalent. If the amount of glucose present in the incubation mixture exceeded 50 µg, the assay was repeated with a more diluted sample of the enzyme solution.

(b) Occurrence of Invertase Activity in Various Tissues of Adult Simuliids

An attempt was made to determine whether invertase activity was localized in the midgut or was also present in other tissues of the blackfly. Various tissues of sugar-fed females of *P. fuscum* and *S. venustum*, both 5 - 10 days old, were assayed for invertase activity (Table 21). No invertase activity was detected in the salivary glands of either species. Weak invertase activity found in extracts of fully grown eggs in the ovaries of *P. fuscum* females and in carcasses, carcass wash and residual fluid of both species, is unexplained at present. However, there is a strong possibility that during dissection of those tissues in a dissecting medium contamination by midgut contents may have occurred. Strong invertase activity was always found in the midgut of both species. The crop, which receives sugary material and stores it temperarily, showed no sucrose-hydrolysing activity when the crops of water-fed and sucrose-fed females of both species were assayed for invertase activity *in vitro*. However, a large quantity of glucose was contained in the crops of sucrose-fed females.

When the crop contents of S. venustum females given sucrose for two days were chromatographed, an almost equal quantity of fructose, glucose and sucrose was observed on the chromatogram.

(c) The Effects of Incubation Time, pH and Homogenate Concentration upon Invertase Activity

To determine the relation of incubation time and degree of sucrose hydrolysis by invertase action, two pools of homogenates were prepared from S. venustum females and were adjusted to contain 0.6 fly per ml. For 90 min of the incubation period at 37°C, the concentration of glucose products in the reaction mixtures and the incubation time showed a linear relationship (Graph 15), indicating that the products of sucrose hydrolysis did not affect invertase action.

The optimum hydrogen ion concentration for black-fly invertase activity was determined with homogenates of midguts and whole flies of S. venustum females. The homogenates were diluted with various pH buffer solutions to contain a concentration of about 0.5 midguts/ml or 2.5 flies/ml. The relative invertase activity at various pH values is shown in Graph 16. The activity of invertase from the two homogenates was practically identical, showing that the enzyme was capable of acting in a weakly acid medium. In both homogenates with maximum invertase activity occurred pH 6.2.

Although a purified invertase material from black-flies was unobtainable with our method, an attempt was made to determine the effect of enzyme concentration upon the action of sucrose hydrolysis. The homogenates of S. venustum females were mixed with phosphate buffer at pH 6.2 to give a series of dilutions (Graph 17). Each of the diluted homogenates was regarded as diluted enzyme concentration. In two pools tested, the

amount of glucose products (by sucrose hydrolysis) formed during a 1-hr incubation was proportional to the number of flies in the homogenates at least to 1.4 flies/ml, the maximum concentration tested (Graph 17).

(d) Invertase activity in water-fed and sugar-fed simuliids

Although the principal food of female black-flies is blood, male and female simuliids both feed extensively on nectar of which the principal constituents are fructose, glucose, sucrose and some other di- and tri-saccharides (Wykes, 1952). Sugar alone is sufficient to maintain adult life and the flies were able to live for several weeks in the laboratory on sucrose solution. Therefore, experiments were undertaken to determine whether the feeding of sucrose, which is the main substrate for invertase, stimulates an increased invertase activity in the flies. Both sexes of newly emerged S. venustum were divided into two groups, one of which was given only water for 24 hr and the other provided with dry sucrose and water for 72 hr. The homogenates prepared from these flies were assayed for invertase activity. The results indicate that ingested sucrose did not specifically stimulate the enzyme activity in either sex (Table 22). In females, a water-fed fly was able to hydrolyse about 0.04 µM sucrose per min, but a sugar-fed fly about 0.05 µM sucrose per min. The enzyme activity in non sugar-fed and sugar-fed females were compared by Student's "t" test, to check whether the mean increase in sugar-fed females was significant. The P value was 0.19 which indicates the sugar feeding did not stimulate invertase activity.

In males, the action of invertase in sugar-fed flies was similar to that in water-fed flies, being 0.039 µM sucrose hydrolysis/min for a water-fed male and 0.036 µM sucrose hydrolysis/min for a sugar-fed male.

(e) Invertase Activity in Female Simuliids after Feeding on Blood

The following experiments were undertaken to determine whether blood feeding increases the invertase activity in black-flies. Homogenates from females of S. venustum which had fed on human blood and been kept at 18-20°C were prepared at various intervals after the blood meal. Assay of invertase activity was made from three different pools of flies which were homogenized at each time interval and the results were averaged (Table 23). The invertase activity immediately after a blood meal sharply increased and remained almost constant for the 48-hr test period, although a slight decrease was noticed 14 hr after the blood meal. The flies dissected 48 hr following the blood meal revealed no advanced digestion of blood in the ventriculus.

(f) Oligosaccharide Synthesis by Black-Fly Invertase

While determining the effects of pH and enzyme concentration on invertase activity, samples of the incubation mixture were chromatographed to observe whether black-fly invertase was capable of synthesizing oligosaccharide(s). No sugars, other than fructose, glucose and sucrose, could be detected on the chromatograms. The concentrations of invertase and substrate used in the previous experiments were both considered to be too low to permit oligosaccharide synthesis, and any oligosaccharide formed (if synthesis occurred) would be difficult to detect on the filter paper. Therefore, the following tests were carried out at a higher concentration of enzyme and substrate. Homogenates were prepared from S. venustum females in the concentration of 10 flies per ml. Similarly homogenates were prepared from midgut, crop and salivary gland of female and midgut of male, at a concentration of 10 tissues per 0.1 ml. Then 0.02 ml of each homogenate was incubated in 0.08 ml of 2% buffered sucrose solution for 1 hr at

37°C. Two controls were used, (i) boiled homogenates substituted for active ones and (ii) sucrose replaced by glucose and fructose solution. After incubation further reaction was stopped by placing the tubes containing the incubation mixtures in a boiling water bath for 10 min after which these were chromatographed.

In control (i), there were no other sugars except sucrose and in control (ii), glucose and fructose were observed on the filter paper. No oligosaccharides were detected from the reaction mixtures of salivary glands and crops. An oligosaccharide was, however, detected in all five tests of female midguts, four tests of male midguts, and six tests of whole females (Fig. 53).

DISCUSSION

Feeding Habits and Role of Buccal Sensilla

Haematophagous simuliids are known to take two types of food, blood and nectar (Edward, 1915; Bequaert, 1934). Blood is the essential diet of most female black-flies for reproducing their successive generations, while nectar, which consists mainly of sugars, is the usual food for male flies but is also imbibed by females as an energy source (Hocking, 1953).

Although Wenk (1965) was able to induce many females of Boopthora erythrocephala (De Geer), a mammalophilic European simuliid, to feed on humans, rabbits or "naked" blood in the laboratory, little success has been achieved in inducing large numbers of Ontario simuliids to feed on hosts in captivity (Davies and Peterson, 1956; Wood and Davies, 1966). S. venustum females were reluctant to feed on whole blood in the laboratory, but with the addition of sucrose solution, many were induced to feed on blood-sucrose mixtures. However, to induce large numbers of flies to engorge fully, a sucrose concentration of more than 50 percent was needed. The order of preference for three different types of blood, each containing an equal volume of sucrose solution, was found to be human>duck>chicken, based on the number of flies which fed and how much each imbibed. These experimental results agree with observations in nature that S. venustum females are attracted mostly to mammals, seldom feed on duck and rarely, if ever, on chickens (Davies and Peterson, 1956; Davies, unpublished notes).

Induced feeding with blood-scurose mixtures, however, failed to

promote egg development in the females, whether females were fully engorged with 50% blood or partly engorged with 70% blood in the sucrose solution. This suggests that, to initiate and complete egg development, the blood proportion of the blood-sucrose mixture in the midgut must be above a certain threshold value and the amount of blood, or possibly of the mixture, in the midgut must be sufficient to trigger a neuro-secretory sequence and thus initiate vitellogenesis as is suggested for other bloodsucking insects (Detinova, 1953, 1962; Larsen and Bodenstein, 1959).

Whether ingested blood goes first to the crop or to the midgut in female black-flies has been open to speculation. Patton and Evans (1929) believed that blood ingested by Simulium females went directly into the crop and then was immediately dispatched into the midgut. A similar report was made by Vargas (1942), who referred to blood entering the crop of S. ochraceum Walker. On the other hand, both Blacklock (1926a,b) and Lewis (1953) found no blood in the crop of S. damnosum. This was substantiated in the present study for the following species: P. decemarticulatum, S. aureum, S. croxtoni, S. latipes, S. quebecense, S. rugglesi, S. venustum and S. vittatum. None of the hundreds of blood-engorged females dissected nor the over 300 of them examined by serial sections showed blood in the crop. On the other hand, sucrose solutions fed to black-flies in the laboratory were always found in the crop.

Our experimental data on the competitive action of blood and sucrose in females of S. venustum suggested that the distribution of blood-sucrose mixtures in the digestive organs of black-flies was determined in part by the concentration of sucrose used. When sucrose concentration in the blood exceeded 0.4 M, most of the food was sent into the crop rather than into the midgut. On the other hand, when the sucrose concentration was less

than 0.2 M in the blood, more of the food passed into the midgut than into the crop. Thus with about 0.3 M sucrose in the mixture almost equal amounts went to the crop and midgut. When the sucrose concentration in the blood decreased to less than 0.02 M, the mixture went exclusively to the midgut. However, less blood-sucrose mixture was imbibed at lower sucrose concentrations in blood. Wenk (1965) observed that in females of B. erythrocephala, pure dextrose solution passed into the crop, while a concentration of 25-75% blood in a 20% dextrose solution appeared to be sufficient to pass partly or completely into the midgut. A similar phenomenon in mosquitoes has been reported by several workers. Bishop and Gilchrist (1946) stated that in A. aegypti it was the nature of the food and not the method of feeding which determined whether the food went into the stomach or diverticula, and Trembley (1952)showed that the switching mechanism was not as well developed in Anopheles and Culex as it was in Aedes mosquitoes. Day (1954) pointed out that the relative concentrations of blood and sugar in blood-sugar mixtures determined whether the food went into the ventral diverticulum or midgut of A. aegypti. Indeed Hosoi (1959) suggested that, for C. pipiens var. pallens Coq., even a slight change in the relative concentration of such mixtures would be appreciated by the mosquito.

In the present investigation various types of sensilla were observed on the ventral (epipharyngeal) surface of the labrum and in the cibarial pump of 16 species of Ontario simuliids. Since the sensilla, which were innervated from the labral nerve and frontal ganglion, were directly in contact with the food which passed through the food channel made by the labrum and the hypopharynx, it was considered that they would act as taste receptors. Emery (1913) who first noticed two circular

spots in the cibarial pump of S. vittatum, called them gustatory sensilla. Gibbins (1938), however, believed that the numerous spines on the ventral surface of the labrum-epipharynx formed an effective trap for wandering microfilariae of Onchocerca. Nicholson (1945), in comparing the mouthparts of S. venustum and Cnephia dacotensis, noticed a number of pegs on the labrum and two spots in the cibarial pump of both species and that they were sensilla. Buerger (1967) observed these structures in S. venustum and S. vittatum. However, she referred to the penultimate pair of sensilla near the tip of the labrum ventrally as being campaniform, whereas in the present study fine spines were found which are easily detached during clearing with KOH. Wenk (1962) traced the innervation of the various sensilla and called them gustatory organs. In mosquitoes similar sensilla were found near the tip of the labrum (Robinson, 1939; Christophers, 1960; von Gernet and Buerger, 1966) and in the cibarium (Day, 1954; Christophers, 1960; von Gernet and Buerger, 1966).

The function of the labral and cibarial sensilla may be twofold, first for the continuation or termination of sucking, and second for regulating whether the food goes to the midgut or the crop. Day (1954) suggested that the discrimination of blood and sugar in A. aegypti was performed by sensilla in the buccal cavity which discharged impulses through the stomogastric system causing relaxation of the cardiac sphincter in the case of blood and of the muscle fibers at neck of the ventral diverticulum in the case of sugar. Furthermore, von Gernet and Buerger (1966) identified two types of sensilla on the tip of the labrum of 21 species of mosquitoes as well as reconfirming the presence of cibarial sensilla, and suggested that blood and sugar may be first distinguished by the labral sensilla and sucking initiated. When the food entered the cibarial pump, the various

sensilla were excited and discharged impulses to the muscles controlling the cardiac sphincter. Other workers agreed that there were chemosensory organs on the tip of the labrum of mosquitoes (Robinson, 1939; Christophers, 1960). However, Hosoi (1959) with C. pipiens and Owen (1963) with Aedes dorsalis (Meigen) and Culiseta inornata (Williston) disagreed, believing that the sensilla on the tip of the mosquito labrum were not chemosensory and did not affect the sucking of blood or sugar solutions. On the other hand, they agreed with Day (1954) and Christophers (1960) that the cibarial sensilla were chemosensory. Hosoi (1959) showed that adenosine-5-phosphates of the red blood cells afforded the main stimulus for gorging on a blood meal.

Salivary Glands and Crop

It is generally known that in most blood-sucking Diptera saliva is important in maintaining blood in a fluid state for transport to the gut. Numerous studies have been made on the salivary glands of bloodsucking insects, but surprisingly few on those of black-flies. Experiments indicated that an anticoagulant was present in the salivary glands of S. decorum females, and the demonstration of the anticoagulant in the saliva of S. venustum and S. vittatum agreed with the work of Hutcheon and Chivers-Wilson (1953) who showed such a factor in extracts of thoraces of the above species. Anticoagulants have also been reported in various species of mosquitoes (Cornwall and Patton, 1914; York and Macfie, 1924; Metcalf, 1945; Hudson, 1964) and of tsetse-flies (York and Macfie, 1924; Lester and Lloyd, 1928; Hawkins, 1966). At present the nature of the anticoagulant in black-fly salivary glands is unknown. Fairbairn and Williamson (1956) carried out extensive tests on the saliva of tsetse-flies and concluded that the anticoagulant factor was a substance of low molecular

weight, probably adsorbed on a protein. The factor was probably not heparin since all tests for acid polysaccharides were negative. Histochemical tests for acid mucopolysaccharides in the salivary glands of Aedes aegypti were also negative (Orr et al, 1961). A heparin-like substance has been found in the salivary glands of Tabanidae and Reduviidae (Markwardt et al 1959, 1960). These authors described the factor as being similar to hirudin found in leeches. Both heparin and hirudin inhibit thrombin in the blood and therefore prevent the formation of fibrin from fibrinogen.

Our investigations showed the presence of agglutinin in the salivary glands of S. decorum, S. venustum and S. vittatum females. However, newly emerged S. decorum females possessed no agglutinin, suggesting that the factor had not yet developed. Moreover the salivary glands at the time of emergence were smaller than older ones. In female mosquitoes also immediately after emergence the glands are small, thin and unconvoluted (Orr et al, 1961), and no agglutinin appeared until 8-12 hr after emergence (Metcalf, 1945). The fact that agglutinin and presumably anticoagulant were absent in the newly emerged female black-fly and that they did not develop for at least 12-24 hr after emergence, was consistent with the observation that females of S. venustum did not suck blood from hosts within 24-48 hr after emergence (Davies and Peterson, 1956, p. 627). No agglutination of cow blood cells occurred when they were treated with extracts of S. decorum and S. venustum females, nor of chicken blood cells in repeated trials with S. decorum extracts. For saliva of Anopheles maculipennis, York and Macfie (1924) reported negative agglutination with the blood of monkey, guinea pig and mouse, while de Buck (1937) obtained positive results with this mosquito and the same species of mammals. Chicken blood gave negative results with

A. quadrimaculatus Say (Metcalf, 1945), while A. maculipennis Meig. gave positive results (de Buck, 1937).

It is generally believed that the saliva of simuliids is toxic (Rempel and Arnason, 1947; Paulovskyi, 1948; Hutcheon and Chivers-Wilson, 1953; Usova, 1961; Fallis, 1964); the toxin is neutralized by alkali and does not lose its toxicity when heated to 100°C for 2 hr (Usova, 1961). The exact nature of the toxic material is unknown although Hutcheon and Chivers-Wilson (1953) detected from extracts of head and thorax of the female S. venustum and S. vittatum low concentrations of histamine which might be responsible for some of the toxic manifestations. Brues (1946) believed that the salivary glands of the black-fly secrete a powerful proteolytic enzyme which causes a minute haemorrhagic spot on the biting site. However, in our studies no such enzyme was detected.

Wanson (1950) reported that the crop content of female S. damnosum showed a strong anticoagulant activity on human blood in vitro and injection of the crop content into the human skin caused a reaction which was similar to the one caused by the bite of the female. Thus he believed that the salivary fluid was accumulated in the crop. It was also suggested by Davies and Peterson (1956) that early in the season the black-fly bite of S. venustum may introduce more salivary fluid, accumulated in the crop, into the wound by pressing the crop at the beginning of a feeding, which causes more bleeding than with the flies later in the season. The contents of five crops of S. venustum females which were collected in late spring showed no anticoagulant factor. Indirect tests for haemagglutinin in the crops were also negative both in vitro and in vivo. In vivo tests were made by feeding S. venustum females with 10% blood cells in 0.2 M sucrose solution and examining the blood cells in the crops at 10 to 30 min later.

However, black-flies earlier in the season might have revealed a different result. Yorke and Macfie (1924) were unable to find an agglutinin factor in the emulsions of the ventral diverticulum or posterior midgut of A. maculipennis. In Glossina, however, salivary fluid seems to be present in the crop. Lester and Lloyd (1928) showed that removal of salivary glands from G. tachinoides Westwood and G. morsitans Newstead resulted in blood clotting in both the proboscis and the crop. Hawkins (1966) demonstrated that extracts from both the salivary glands and crops of tsetse flies possess fibrinolytic and anticoagulant activity to the same extent, and believed that the activity in the crop originated from the salivary glands and that it was sucked back into the crop with the blood meal. The feeding mechanism of Glossina is different from simuliids because in the former blood goes first into the crop and then into the midgut, while in the latter it goes directly into the midgut. Although the present results suggest that salivary fluid may not be stored in the black-fly crop or if present, it is not in the active state, tests with different stages of flies after emergence and various concentrations of the crop contents should be carried out before definite conclusions can be drawn.

Lester and Lloyd (1928) reported the presence of a powerful coagulant in the posterior midgut of Glossina and believed that the coagulant neutralized the anticoagulated blood meal and caused a rapid clotting to retain the blood in the proper region while dehydration and digestion took place. Similarly the presence of a coagulant was reported in the wall of the posterior midgut in females of Culiseta annulata (Schrank) and Culex pipiens but not in A. maculipennis (de Buck, 1937). However, Hawkins

(1966), demonstrated that trypsin was responsible for the coagulation observed with the midgut extracts of Glossina, by mixing soybean trypsin inhibitor with the extract and subsequently testing for coagulation.

In the bloodsucking hemipteran, Rhodnius prolixus Stal, the midgut secreted an anticoagulant the properties of which seemed to be different from the salivary anticoagulant (Hellmann and Hawkins, 1964). Further studies of the two anticoagulants with the thrombin-fibrinogen reaction demonstrated that the midgut anticoagulant (prolixin-G) had its activity reduced by different amounts following various treatments; cooling (-20°C) for 24 hours, N/10 HCl, freezing-drying and dialysis, while the salivary anticoagulant (prolixin-S) had no loss of its activity with the above treatments (Hellmann and Hawkins, 1965).

The exact function of the agglutinins in the black-flies is unknown but, in all females dissected immediately after a blood meal, the ingested blood formed a semi-solid mass in the posterior midgut. These changes may be brought about in the ingested blood by agglutination of the red blood cells rather than by the absorption of water or by the action of a coagulant as is the case in mosquitoes (Clements, 1963).

Blood Digestion and Peritrophic Membrane

It might be expected that digestion of blood would proceed more uniformly in insects which thoroughly mixed digestive enzymes into the food bolus. However, it has been shown that in Culex pipiens (Huff, 1934), Aedes aegypti (Stohler, 1957; Christophers, 1960) and Simulium damnosum (Wanson, 1950), the digestion first began next to the midgut wall and proceeded toward the center. A similar process of blood digestion was observed in females of six simuliid species which were maintained at

18-20°C and killed at various intervals after a blood meal. The signs of digestion could be recognized in freshly dissected specimens by the change in the original bright red colour of the blood mass to brown red, and in the sections, by the appearance of the digested product between the peritrophic membrane and the gut wall or of altered blood pigment in the periphery of the blood mass. The digestion always began at the periphery of blood mass, particularly at the posterior end near the pyloric region, and it progressed toward the center and anteriorly, during which the semi-solid blood mass was successively liquified. When the altered blood pigments (waste products) increased in the pyloric region, the waste products with disintegrated peritrophic membrane began to enter the hindgut, while the maturing eggs pushed the ventriculus anteriorly. Unchanged blood cells were found in the anterior center of the blood mass 120-168 hr after a blood meal at 18-20°C. Lewis (1953) noticed that digestion was completed in S. damnosum in less than 72 hr at 23 to 25°C, and Downe (1957) stated that in a few specimens of S. venustum digestion was completed within 60 hr at 19-20°C; however, he judged digestion by precipitin tests which only provided a measure of the rate of digestion serum proteins in the blood meal.

Investigations of digestive enzymes in various tissues of the black-flies indicated that a trypsin-like enzyme was responsible for blood digestion, that the enzyme was secreted by the midgut cells, and that the secretion was greatly stimulated by a blood meal. The mechanism of secretion of the enzyme by the midgut cells is not clearly known. However, Bertram and Bird (1961) on the basis of their electron microscopic studies on the midgut of A. aegypti before and after a blood meal, suggested that

the unfolding of the whorls of the endoplasmic reticulum in the gut cells was probably related to the separation of proteolytic enzymes from the cell contents and their conduction to the luminal surface, and that was possibly also related to absorption of the products of digestion.

A peritrophic membrane formed around ingested blood in adult females of six species of black-flies. Soon after a blood meal, there appeared a viscous substance around the blood mass. The substance gradually solidified and became a membrane. The hardening first began on the periphery of the viscous substance near the gut wall and progressed inward. If the hardening of the substance was well advanced, the membrane was easily detachable from the midgut wall but firmly attached to the blood mass. As the volume of the digesting blood decreased, the membrane, still firmly attached to the blood mass, became markedly shrunken and well separated from the gut wall. When digestion was advanced and the waste products formed around the blood mass within the membrane, the solidified membrane seemed to soften, separating from the blood mass or disintegrating and becoming incorporated with the altered blood pigments, which were eventually eliminated into the hindgut. As long as the membrane was in contact with undigested blood mass, it appeared to remain intact. Such an intact membrane was observed around the anterior portion of the undigested blood mass, while the membrane around the posterior part of the digested mass had disintegrated.

After the waste products and the disintegrated or disintegrating peritrophic membrane had been completely excreted, there remained in the midgut some material, "old gut content", which had been accumulated as a viscous substance on top of the anterior blood mass and solidified during the period of digestion. The "old gut content" contained various gritty

particles and occasionally yeast-like material which may have been derived from the crop or been ingested after blood feeding. If there were many particles, the viscous substance often formed a membrane which enveloped the particles, thereby separating the particles from the blood mass. The "old gut content" was called the old peritrophic membrane residue by Lewis (1953) who believed that a part of the peritrophic membrane from previous blood feeding (presumably from the posterior midgut since he believed that no peritrophic membrane secretion occurred in the anterior midgut) remained after the blood had been digested. However, in our studies, such an "old gut content" was observed in females of S. decorum and S. vittatum which had emerged from pupae in the laboratory and had taken no previous blood meal. It appears, therefore, that the "old gut content" of unfed females and of females which had completed the digestion of a blood meal were formed in the same place (the anterior tubular midgut) and in the same way. If we consider the secretion of the anterior tubular midgut to be the same material as the peritrophic membrane of the posterior midgut, the "old gut content" could be called a part of the old peritrophic membrane residue as Lewis (1953) suggested.

When females of S. venustum and S. vittatum fed on a mixture of blood and sucrose which went directly to the midgut, a peritrophic membrane formed around the mixture regardless of the size of the meal. However, when the mixture went directly to the crop and was slowly dispatched into the midgut, no peritrophic membrane was observed in the midgut. It seems that, if the blood-sucrose mixture in the crop is constantly sent in small amounts to the midgut, there would be no increase of secretion of PM substances or the secreted PM substances would blend with the mixture being

sent from the crop and be digested without forming a membrane. However, more critical tests are necessary to clarify whether an interval after ingestion of the mixture is required before the formation of the peritrophic membrane or whether some other factors are needed to induce the formation of the membrane.

Lewis (1953) observed no peritrophic membrane in newly emerged or sugar-fed females of S. dammosum. Similarly Stohler (1957) examined several newly emerged and sugar-fed females of A. aegypti without finding the membrane in the midgut. However, in the present study, materials similar to that of the peritrophic membrane was found in the anterior tubular midgut of sucrose-fed females of S. decorum, the initiation of the "old gut content".

Bertram and Bird (1961) indicated that the microvillar surfaces were involved as the source of the secretion forming the peritrophic membrane in A. aegypti. Within half an hour of ingestion of blood, copious fine particulate material which was to form the membrane appeared between the microvilli, extending from their bases to beyond their tips. This was at a time when unfolding of the whorls of endoplasmic reticulum had just begun, and Bertram and Bird believed that the membrane was secreted directly from microvilli of the epithelial cells without close dependence on the endo-plasmic reticulum.

For Glossina, which produces the peritrophic membrane from a ring form of the proventriculus, Wigglesworth (1929) stated that increased membrane formation was stimulated by a blood meal and that in the newly emerged fly the membrane was ragged and discontinuous. However, Willett (1966), on the basis of his thorough observations of the peritrophic

membrane in teneral flies of eight species, concluded that the membrane was present as an unbroken sac before feeding, the sac growing larger as the fly aged.

Until recently the peritrophic membrane was regarded as protecting the midgut cells from abrasion by hard particles in the food, and was thought to be present only in those insects which ingest hard food (Day and Waterhouse, 1953). This belief was supported previously by finding no membrane in many insects which feed on fluids or blood. However, peritrophic membranes are being found in many insects in which they were formerly believed to be absent (Wigglesworth, 1965). Likewise, despite the general belief that there were two origins of peritrophic membrane in insects, one from a ring of ectodermal cells situated in proventriculus and the other from the midgut epithelium by periodic delamination, there is a strong belief that peritrophic membrane formation in certain insects, e.g. adult mosquitoes and simuliids, may combine the two origins, and that this may represent a stage in the evolution of the second type from the first (Wigglesworth, 1965).

Digestive Enzymes

(a) Trypsin-like Enzyme

In many species of Ontario simuliids, the females are haematophagous requiring a blood meal as nutrient for the second and subsequent ovarian cycles, and often for the first (Davies et al, 1962). In the present study a trypsin-like enzyme was demonstrated in seven species of simuliids, using p-tosyl-L-arginine methyl ester HCl (TAME) as a specific substrate. When various tissues were tested separately, trypsin activity was found to occur mainly, if not wholly in the midgut of the

adult simuliids. This finding was further supported by the fact that the enzyme activity from the midgut of S. venustum females increased after the flies were fed on blood-sucrose mixtures. Wagner et al (1961) similarly found that in unfed A. aegypti most of the protease was localized in the midgut when this and other residue were tested separately for the enzyme activity.

In the black-fly species examined, no trypsin activity was found in extracts of salivary glands. This agrees with previous reports of no proteinase activity in the salivary glands of blood-sucking Diptera, such as Glossina and Chrysops (Wigglesworth, 1929, 1931), A. aegypti (Metcalf, 1945; Fisk, 1950) and Stomoxys (Rostom and Gamal-Eddin, 1962). Moreover in the black-flies, no trypsin activity was found in the crop even when it was filled with blood-sucrose mixture, indicating that it is probably a mere storage organ for ingested food, and that digestion takes place only in the midgut.

Although it is generally known that in animal tissues there are proteins which inhibit trypsin activity (Karlson, 1963), this appears not to be true of black-fly tissues. The trypsin activity determined with midgut alone differed little from that of homogenates of whole simuliid bodies. Therefore, in black-fly body tissues, either there were no proteins which inhibit trypsin, or, if such proteins were present, the quantity was too small to affect determination of trypsin activity.

The pH optimum for trypsin activity in the homogenates of S. venustum females fed on human blood-sucrose mixture and of S. rugglesi females fed on duck blood are almost identical, which suggests that the enzymes in these two species are similar. However, S. rugglesi contained considerably more

trypsin activity than does that from S. venustum. The pH optimum of the enzyme from these two species, 8.4, agrees with the report of Wanson (1950) that in S. damnosum tryptase (trypsin) was almost active at a pH of about 8.5.

The level of trypsin activity determined in sucrose-fed black-flies at intervals after they emerged from the pupae differed in various species, but the enzyme activities 1 hr after emergence and 24 hr later were almost identical. These results suggest that trypsin was present before emergence or at least at the time of emergence, and that sugar feeding did not influence the enzyme activity. Similar results were reported for the Calliphora midgut which showed a low proteinase level on a sugar and water diet but increased after a protein meal (Thomsen and Møller, 1963).

S. venustum (a mammalophilic, anautogenous species) had a higher trypsin activity than S. vittatum (mammalophilic and autogenous for the first gonotrophic cycle) and P. decemarticulatum (ornithophilic and anautogenous). Also these three species possessed a somewhat higher trypsin activity than P. fuscum (mammalophilic and autogenous for first gonotrophic cycle). C. dacotensis (a non-bloodsucking species with reduced mouthparts unsuited for piercing the vertebrate skin) retained a higher enzyme activity than certain haematophagous simuliid species. Downes (1958) believed that Diptera were originally bloodsucking, and that non-bloodsucking forms are a secondary development; high trypsin activity in C. dacotensis supports his belief. Presumably when C. dacotensis evolved into an autogenous species, there followed a reduction in the female mouthparts but little decrease in the basic trypsin production. One might speculate in the same way for male simuliids and culicids. In the present study male simuliids had levels of trypsin activity similar to sugar-fed females; Wagner et al (1961) found

both sexes of A. aegypti to have similar proteinase activity before blood feeding. Simuliid males, unlike females, showed no rise in enzyme activity after they fed on a blood-sucrose mixture. It may be that males of simuliids and culicids were originally haematophagous, or at least fed on proteins. Of course, the larvae of non-bloodsucking simuliid species and males of haematophagous species may secrete midgut trypsin to digest their food and this secretory activity may be retained in part by these adults.

Other workers have searched for biochemical differences between species of mosquitoes and other insects as an aid in taxonomy. Between various mosquito species quantitative differences were found in amino-acid composition (Micks and Ellis, 1951; Ball and Clark, 1953; Micks and Gibson, 1957), vitamin content and other biochemical properties (Micks et al, 1959). The differences in trypsin activity demonstrated in this study between various black-fly species, with further extension, may prove of use in taxonomy. Recently the possibility of enzymes playing an important role in animal taxonomy was reviewed by Wilson and Kaplan (1964).

In females of S. rugglesi and P. decemarticulatum which were fed on blood of different birds and kept at 18-20°C the trypsin activity greatly increased during a 24-hr digestion period, but with a different pattern of increase for each species. However, the maximum enzyme activity may not have been reached during this period. In P. decemarticulatum, the enzyme activity was much greater about 96 hr after a blood meal than 24 hr after, and in S. quebecense, the activity was greater after 72 hr than after 1 hr. Fisk and Shambaugh (1952) reported that maximum proteinase activity in A. aegypti occurred about 18 hr after a blood meal at 27°C, and Akov (1965) reported a maximum activity between 18 and 24 hr at 28°C, whereas Gooding (1966b) found that the peak was reached only after 24 hr 36 hr at about

26°C. In C. fatigans at about 26°C the maximum activity occurred 36 hr after the females fed on blood (Gooding, 1966b), and in the bloodsucking muscid, Stomoxys calcitrans about 13 hr after a blood meal (Champlain and Fisk, 1956). In the two black-fly species studied, high trypsin activity was probably sustained for a longer period than 24 hr since these species were maintained at low temperature after blood meal. This assumption was further supported by histological observations in which some undigested blood was found in both species 120-168 hr after a blood meal. This agrees also with the report of Fallis (1964) that, in S. rugglesi, blood digestion was completed in 96-144 hr at 19-20°C. O'Gower (1956) demonstrated that, under the same conditions, the period of complete digestion of proteins in a given population differed in various species within the same genus of mosquitoes. In black-flies temperature influenced the production of trypsin, thus lowering digestive activity indirectly (See Fig. 7 and Results IIIb). However temperature would also affect the rate of digestion directly. Also Shlenova (1938) reported that temperature and humidity greatly affected the rate of blood digestion in mosquitoes. Although O'Gower (1956) found that the rate of digestion in females of Aedes notoscriptus Skuse could be increased by reducing the exposure of the females to light, Gooding (1966b) reported that holding adults of A. aegypti and C. fatigans in continuous light or darkness had no effect on the level of proteinase activity in their midguts. Thus the time for complete digestion of blood and the attainment of maximum enzyme activity after a blood meal may differ with the species, type of blood fed, and physical factors such as, temperature, humidity and perhaps photoperiod.

Thomsen and Møller (1959) showed that the oesophageal nerves of the blow-fly transported neurosecretory material from the brain to the

midgut wall and that interrupting this transport resulted in a reduced proteinase activity in the midgut. They (1963) further showed that the development of proteolytic enzymes in the midgut of this insect was controlled by the median neurosecretory cells in the brain. Langley (1965) reported that in Glossina the distensible crop and the proventriculus are potential sites for the production of stimuli which might control neuroendocrine function through which tsetse flies would be able to produce proteolytic enzymes in response to the distension of the crop with the blood meal.

Whole blood diluted in sucrose solution, a mixture which first goes to the crop, apparently stimulated an increased trypsin activity in S. venustum females. The level of increase in enzyme activity seemed independent of the concentration of blood in the blood-sucrose mixture, but was related to the amount of mixture ingested. The distension of crop by ingestion of the mixture may have been responsible for the production of the enzyme in the midgut of the females. However, females fed on sucrose solution alone, with greatly distended crops, showed no increase in enzyme activity. Our results on this aspect are similar to Langley's (1966a) studies on Glossina. He reported that the production of proteinase in the midgut of Glossina depended on the volume of blood-saline mixture ingested rather than the concentration of blood in the meal, and that flies fed on saline alone failed to increase the level of enzyme activity above that in unfed flies. On the basis of his results, Langley (1966b) postulated that the production of proteinase was controlled hormonally through the distension of the crop acting on the neuroendocrine system, but the presence of serum was necessary for the production of enzyme in the midgut. However, in females of S. venustum,

blood corpuscles as well as whole blood were capable of increasing the trypsin activity.

It is of interest that, in the black-fly, trypsin activity was almost identical whether the fly fed on whole blood or blood cells suspended in sucrose solution. However, Shambaugh (1954) with Aedes mosquitoes and Langley (1966a) with Glossina reported that proteinase activity was less stimulated by blood cells suspended in saline than by serum or whole blood. The discrepancy between these findings may be because of species differences or because in the simuliids the erythrocytes were suspended in sucrose solution rather than isotonic saline. However, it is perhaps reasonable that erythrocytes in 0.3 M sucrose solution stimulated trypsin activity in simuliids. Gooding (1966a) reported that the proteinases of Aedes and Culex mosquitoes hydrolysed a haemoglobin preparation much more rapidly than they did the serum proteins in vitro. Haemoglobin, which is the most abundant protein in the blood, is normally found entirely within the erythrocytes, from which it may be released by hemolysis of the cells.

Fisk and Shambaugh (1952) and Shambaugh (1954) suggested that different foods stimulated different levels of enzyme activity in Aedes mosquitoes, which favoured action of a secretogogue as the mechanism of enzyme secretion. However, this assumption seems unsuited to the black-fly. In females of S. venustum fed on different foods, such as whole blood or blood cells from humans, ducks or cows, the trypsin activity showed little variation. Dadd (1961) has shown that in Tenebrio "inert food", such as cellulose powder or water, could cause an increase in the midgut proteinase activity of the same order as that caused by flour.

Thus it appears that the mechanism of enzyme activation in insects may be different from species to species.

(b) Pepsin-like Enzyme

A pepsin-like enzyme, which is a protein-cleaving enzyme like trypsin but active at acidic pH, is regarded as uncommon in adult insects (Wigglesworth, 1965). Although no such enzyme has been reported in adult bloodsucking Diptera, for the sake of completeness in this study, a black-fly species was assayed for this enzyme activity. No pepsin-like activity was found in males or females of the black-fly, P. fuscum. No such enzyme was detected in blood-fed adults of Stomoxys (Champlain and Fisk, 1956) or of Glossina (Langley, 1966a). However, pepsin-like enzymes have been reported in larvae of the house-fly (Greenberg and Paretsky, 1955) and Stomoxys (Lambremont et al, 1959), and in adult females of Calliphora (Fraser et al, 1961).

(c) Amylase

Amylases, which catalyze the hydrolysis of polysaccharides such as starch, glycogen, amylose and amylopectin, are believed to be uncommon enzymes in the midgut of haematophagous Diptera the main diets of which are proteins and disaccharides. This belief was supported by the observations that only weak amylase activity was detected in the midgut of Glossina (Wigglesworth, 1929), of Chrysops (Wigglesworth, 1931), and of Simulium damnosum (Wanson, 1950), and no amylase activity in the midgut of Aedes aegypti (Fisk and Shambaugh, 1954). The present results on amylase activity in S. venustum, S. vittatum, Culex pipiens and A. aegypti further support the belief. Determination of starch-hydrolysing activity in the midgut of these insects indicated that such activity was neglegible. Amylase activity was found to be absent in the salivary glands, crop and ovaries

of these four species. Similarly absence of amylase activity was reported from the salivary glands of Anopheles quadrimaculatus (Metcalf, 1945), Glossina (Wigglesworth, 1929) and Chrysops (Wigglesworth, 1931).

Although neglegible amylase activity was found in the midgut of unfed or sugar-fed black-flies and mosquitoes, it has been a question whether or not blood feeding increased amylase secretion in the midguts of these haematophagous insects. No information is available on amylase activity in blood-fed insects. Fisk and Shambaugh (1954) suggested that, although no amylase activity was found in the midgut of unfed females of A. aegypti, significant amylase activity might have been demonstrated in the blood-fed mosquitoes. Our results on enzyme activity in A. aegypti at intervals after a blood meal showed that the pattern of increasing amylase activity was contrary to that of increasing trypsin activity. Amylase activity increased sharply immediately after blood meal, then gradually decreased to the original level. On the other hand, trypsin activity increased gradually for several hours before decreasing. It seemed quite unlikely that the blood meal had stimulated such sharp increase in amylase secretion in the midgut. The starch-hydrolysing activity in the serum of human blood is well known, the quantity of amylase ranging from 60 to 200 units (Somogyi) in the normal blood serum (Oser, 1965). A comparative study of enzyme activity in host blood and the blood-engorged mosquito showed that the increased amylase activity following a blood meal was actually derived from the host's blood rather than from midgut secretion of the mosquito, and the enzyme activity decreased in accordance with the digestion of blood in the midgut. Similar results were obtained with blood-fed S. venustum females which also showed higher amylase activity

soon after a blood meal followed by a gradual decrease to the level found in sugar-fed flies.

Although little attention has been paid to amylases in the alimentary canals of bloodsucking insects, even less study has been given to such enzymes in other tissues. Our further assay for amylase activity in tissues other then the digestive tract revealed that the highest enzyme activity of the four species occurred in the residual fluid which, in the present investigation, was considered to be mainly haemolymph. Amylases have been found in the haemolymph of many non-bloodsucking insects (Wigglesworth, 1965). It is of interest that in black-flies, S. venustum females, which are anautogenous, showed higher amylase activity that S. vittatum females, which are autogenous for the first cycle of eggs (Davies et al, 1962). In mosquitoes, C. pipiens females which were at beginning of hibernation possessed higher amylase activity than those of A. aegypti which were non-hibernating and from an inbred colony.

The maximum activity of amylase was obtained at pH 6.5 from whole-body homogenates of both males and females of S. venustum. Since the activity of midgut amylase was neglegible compared to that in the residual fluid, this pH optimum would apply to haemolymph amylase. Amylases from various insect species differ widely in pH optima from about 5.5 to 9.5 (Day and Waterhouse, 1953). However, these pH optima refer to amylases in the digestive organs. It is unknown at present whether pH optima of amylases in insect haemolymph show such variation.

The origin and function of amylase in the haemolymph of the black-fly and mosquito is obscure at present, although it may have entered the haemolymph from the larval midgut during larval life or during

metamorphosis in the pupa. Likewise, the function of serum amylase in mammals is unknown, although its origin is believed to be embryonic (Searcy et al, 1966).

No clear correspondence has been found between data on insect amylases and the thoroughly investigated endo- and ecto-amylases originating from other animals and plants. However, the amylase found in the residual fluid of adult simuliids and culicids is probably of the endo-amylase type which seems to occur in insects (Gilmour, 1961) and not one of the exo-amylases which are exclusively of plant or microbial origin (Fischer and Stein, 1960).

(d) Invertase

Invertase activity has been found in the midgut of bloodsucking insects which feed on nectar as well, such as Chrysops (Wigglesworth, 1931) and Aedes mosquitoes (Fisk and Shambaugh, 1954). However, invertase activity is absent from the midgut of Glossina both sexes of which are exclusively blood feeders, while enzymes acting upon carbohydrates (amylase, invertase and maltase) are predominant at times in Calliphora (Wigglesworth, 1929). Although the principal food of female black-flies is blood, males and females both feed extensively on nectar of which the main constituents are fructose, glucose and sucrose, with maltose, melibiose and raffinose occasionally occurring in low concentration (Wykes, 1953).

In the present studies, invertase activity was determined in both sexes of S. venustum and P. fuscum, applying the specific colorimetric assay of glucose with glucose oxidase-peroxidase system. This system was found to be particularly suitable for microanalysis of invertase activity in a small sample. Most invertase activity was concentrated in the midgut of both simuliid species, although some enzyme activity was found in the

residual fluids and carcasses. Glucosidase activity was observed in the blood of the Cecropia moth by electrophoresis (Laufer, 1960). Invertase was reported to be found in the haemolymph of non-bloodsucking insects (Wigglesworth, 1965). It is, however, quite probable that the invertase activity in the black-fly haemolymph and carcasses resulted from contamination by the midgut contents during dissection.

The absence of invertase activity in the salivary glands of the black-flies agreed with studies of other bloodsucking Diptera, such as Glossina and Chrysops (Wigglesworth, 1929, 1931), Anopheles quadrimaculatus (Metcalf, 1945) and Aedes aegypti (Fisk and Shambaugh, 1954). Invertase activity was also absent from the crop and ovaries of both S. venustum and P. fuscum, which substantiates further that the black-fly crop is merely a storage organ. Fisk and Shambaugh (1954), however, reported the presence of invertase activity in the diverticula of A. aegypti. Since they found no invertase activity in the salivary glands of the mosquitoes nor did they believe that the diverticula secreted the enzyme, one might suggest that invertase in the diverticula must have come from another source, e.g. from the midgut by regurgitation. It would be of interest to see whether other digestive enzymes which might have been regurgitated along with invertase are found in mosquito diverticula. However, in our study no trypsin activity was detected in the crops of black-flies which fed on sugar or blood-sucrose mixture.

Adult black-flies are able to live for several weeks in the laboratory on a sucrose solution which is the main substrate of invertase. However, in male and female S. venustum sucrose feeding did not stimulate increased invertase activity, thus the level of the invertase activity was more or less the same whether the black-flies fed on sucrose or not.

Similar results were obtained in the Aedes mosquitoes by Fisk and Shambaugh (1954). Invertase activity in the midgut of A. aegypti showed little increase after a meal of 5% sucrose solution. Black-flies, like mosquitoes, send the sugar solution to the crop first from which the solution is slowly dispatched to the midgut for digestion and utilization. It may be assumed therefore that the quantity of invertase being produced in the midgut before sugar feeding will be sufficient to catalyse the hydrolysis of the sucrose which comes from the crop without any further increase in enzyme secretion in the midgut.

On the other hand, blood feeding increased the invertase activity in S. venustum females. The enzyme activity almost doubled immediately after a blood meal, when compared with that of sugar-fed or non sugar-fed females, and remained almost constant during the 48-hr test period. However, the pattern of increasing invertase activity was in contrast to that of trypsin activity in the same species, in which the trypsin activity increased gradually during this period. Fisk and Shambaugh (1954) found a definite increase of invertase activity in the midgut of Aedes mosquito after a blood meal. Since proteinase activity in the same species of mosquitoes was also stimulated by blood although the pattern of stimulation was different from invertase, they presumed that the secretions of both invertase and proteinase were stimulated by possibly identical factors in the blood meal.

The pH optimum for invertase activity in the homogenates of midgut and whole bodies of S. venustum females were similar, approximately 6.2 in both cases. Thus in the bloodsucking insect, the black-fly invertase appears to have the same capacity for acting in a weakly acid medium as do the gut invertases of non-bloodsucking insects, such as cockroaches (Wigglesworth,

1927; Day and Powning, 1949), blow-flies (Evans, 1956), Trogoderma larvae (Krishna, 1958), Lepidoptera (Srivastava, 1960), Heteroptera (Saxena and Bhatnagar, 1961), pea aphids (Srivastava and Auclair, 1962), Tribolium adults and larvae (Krishna and Saxena, 1962), Hemiptera (Khan and Ford, 1962) and desert locusts (Evans and Payne, 1964).

Reports on invertases from several other sources reveal that two types of invertases have been recognized: β-fructofuranosidase and α-glucosidase termed glucosaccharase or glucoinvertase (Neuberg and Mandl, 1950). The first type of enzyme, present in yeast cells, is considered to attack the fructose end of the sucrose molecule, and it can also act on raffinose which has a free fructosyl unit. On the other hand, glucoinvertase is reported to attack the glucose end of the sucrose molecule and it can act on melezitose having a free glucosyl unit but not on raffinose where the glucose group is blocked with galactose.

Insect invertase is generally regarded as the glucoinvertase type (Waterhouse, 1957; House, 1965), although both types of the enzymes have been demonstrated in the midgut of several insects, such as Calliphora (Evans, 1956), Dysdercus koenigi (Saxena, 1958) and cockroaches (Ehrhardt and Voss, 1962; Banks, 1963).

The concept of the nature and action of invertase has further changed during the past decade as a result of analysis of invertase-sucrose reaction mixtures by paper partition chromatography. Reported information reveals that invertase also synthesizes certain oligosaccharides. Since invertases from yeast (Bacon and Edelman, 1950; Edelman, 1954) and mould (Bealing and Bacon, 1953; Bealing, 1953) catalyse transfer of fructosyl units they are regarded as transfructosidases. On the other hand, invertase from honeydew (White and Maher, 1953) and nectar (Zimmerman, 1954)

transfer glucose groups and are therefore termed transglusidases. Duspiva (1953, 1954) demonstrated that aphid gut invertase synthesized oligosaccharides in vitro and these sugars were also found in the excreta of the aphids. Srivastava and Auclair (1962) similarly reported oligosaccharide synthesis by aphid gut invertase.

The present observations also show that the homogenates of the midgut and whole body of S. venustum and P. fuscum synthesize oligosaccharides. These were detected only when the reaction mixtures had fairly high concentrations of substrate and enzyme. Only sucrose, glucose and fructose could be detected when the reaction mixtures contained a low concentration of substrate and enzyme, 0.028 M sucrose and 0.5 fly/ml respectively.

SUMMARY

When blood and sucrose were given separately, they went to the midgut and crop respectively. When blood and sucrose were mixed, a slight difference in the stimulating power of blood and sucrose in the mixture was distinguished by the flies but the minor stimulus seemed to be overridden.

At least four groups of sensilla (probably contact chemoreceptors) were located in the buccal cavity of the female black-flies. The number of the sensilla, however, varied among the flies. Three groups occurred on the ventral surface of labrum and only one group in the cibarial pump. All the sensilla in the buccal cavity were innervated by the labral nerve which originated from the frontal ganglion. It is suggested that the labral sensilla are important in the distribution of the meal to the crop or midgut.

Occurrence of a peritrophic membrane before and after adult females of several simuliid species fed on blood or blood-sucrose mixture was demonstrated, and its formation and disappearance examined. The origin of "old gut content" (previously thought as residue of peritrophic membrane after first blood meal) was shown to result in large part from the secretion of the anterior tubular midgut before and after blood feeding.

A trypsin-like enzyme was demonstrated in homogenates of six simuliid species in three genera. The enzyme activity was confined mainly to the midgut of the black-flies.

Different levels of trypsin activity were found in sugar-fed females of Prosimulium decemarticulatum, Cnephia dacotensis and Simulium venustum.

Trypsin activity increased after adult female black-flies fed on whole blood, blood-sucrose mixtures or erythrocyte-sucrose mixtures, whole blood giving the greatest increase.

The blood-sucrose mixtures in the crop of female black-flies stimulated steady trypsin activity, as the mixtures were dispatched slowly into the midgut for digestion.

Low temperature depressed the midgut trypsin activity and delayed the blood digestion in the black-flies.

In S. venustum the sugar-fed males had a similar trypsin activity to sugar-fed females, but failed to increase enzyme activity after feeding on a blood-sucrose mixture.

No pepsin-like activity was detected in either sex of the black-fly, P. fuscum.

A high activity of amylase-like enzyme was demonstrated in the residual fluid (mainly haemolymph) of adult females of the black-flies, S. venustum and S. vittatum, and of the mosquitoes, Culex pipiens and Aedes aegypti, but the enzyme activity in their midguts and carcasses was slight.

The marked increase in the amylase activity in the females of S. venustum and A. aegypti after they fed on human blood resulted from amylase activity in the host's blood rather than active secretion by the midgut.

Invertase activity in adult females of P. fuscum and S. venustum was demonstrated by glucose oxidase-peroxidase system. Invertase activity was localized mostly in the midgut when compared with that of other

tissues of the flies.

No increase in invertase was stimulated by sugar feeding in both sexes of S. venustum. Invertase activity increased after blood feeding, but the increase was initially more rapid than found for trypsin, and then remained level.

Oligosaccharide synthesis by the black-fly invertase was observed.

The salivary glands of adult female black-flies contained agglutinin and anticoagulant factors, but these factors seemed to develop at least 12-24 hr after flies emerged from the pupae. No digestive enzymes (trypsin, amylase or invertase) were found in the saliva nor in the crop of adult female black-flies.

The pH optima for trypsin, amylase and invertase in the black-flies were determined to be 8.4, 6.5 and 6.2 respectively.

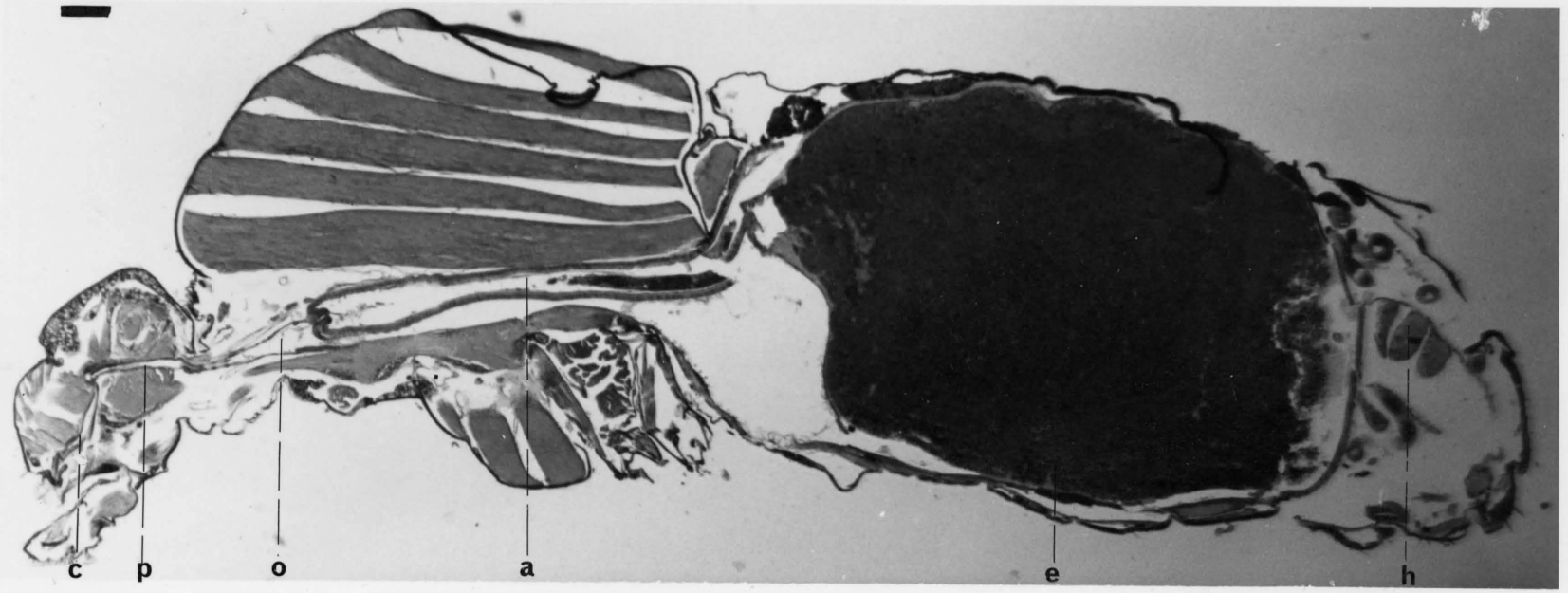
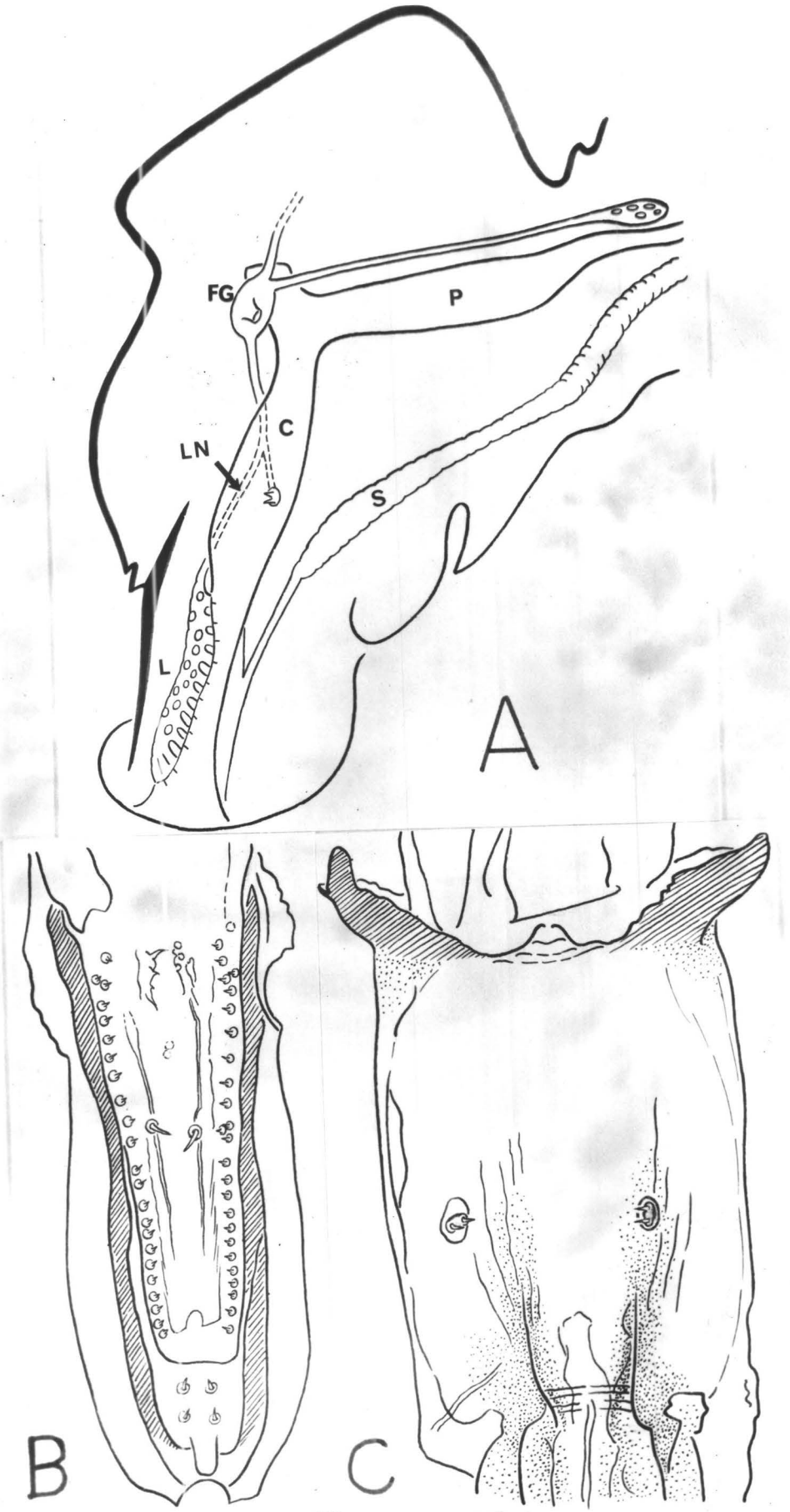


Figure 1. Sagittal section of S.aureum female, 1-2 hr after feeding on chicken blood, showing alimentary canal: c-cibarial pump; p-pharyngeal pump; o-oesophagus; a-anterior midgut; e-posterior midgut with full of blood; h-papilla in the hindgut.

Figure 2. A. Diagram of sensory nervous system in the head and of the location and arrangement of sensilla in the buccal cavity of the adult female black-fly: FG-frontal ganglion; P-pharyngeal pump; C-cibarial pump; L-labrum; LN-labral nerve; S-salivary pump.

B. Ventral surface of labrum showing spine-shaped sensilla.

C. Inner surface of cibarial pump showing a pair of sensilla.



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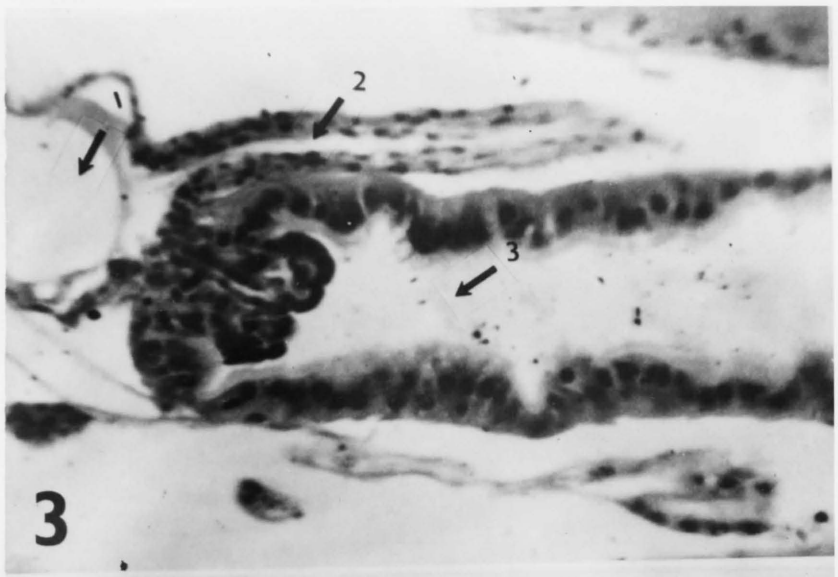
Figures 3-5. Sections of proventriculus and crop duct.

Fig. 3. Longitudinal section (S. vittatum female) 1-oesophagous, 2-crop duct, 3-lumen of anterior midgut.

Fig. 4. Higher magnification of Fig. 3.

Fig. 5. Cross section (S. aureum female). 1-crop duct, 2-cardia, 3-globules discharged by epithelial cells of midgut, 4-lumen of anterior midgut.



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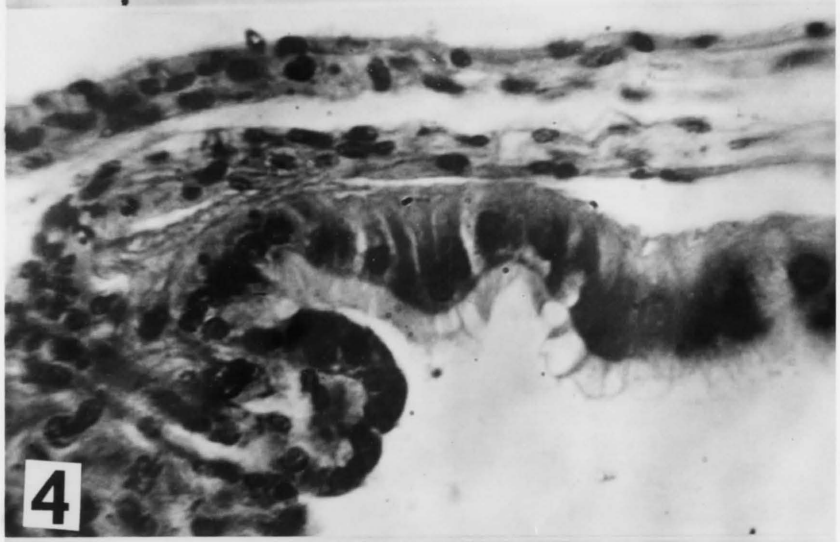

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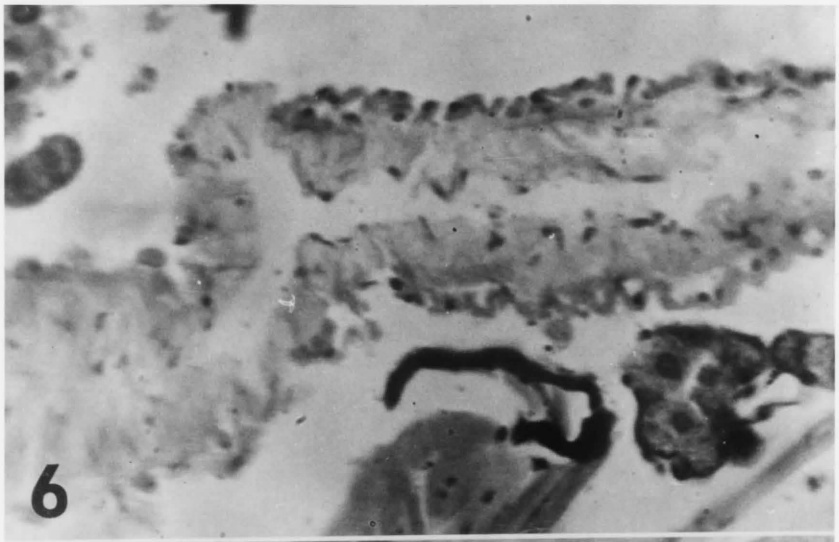
Figures 6-8. Sections of empty crop in black-fly females.

Fig. 6. Longitudinal section of folded crop (S. vittatum).

Fig. 7. Cross section of crop duct near anterior end of anterior midgut. S. decemarticulatum immediately after feeding on blood. Arrow shows the membrane folded.

Fig. 8. Longitudinal section of posterior portion of crop, showing thin epithelial cells (S. vittatum).



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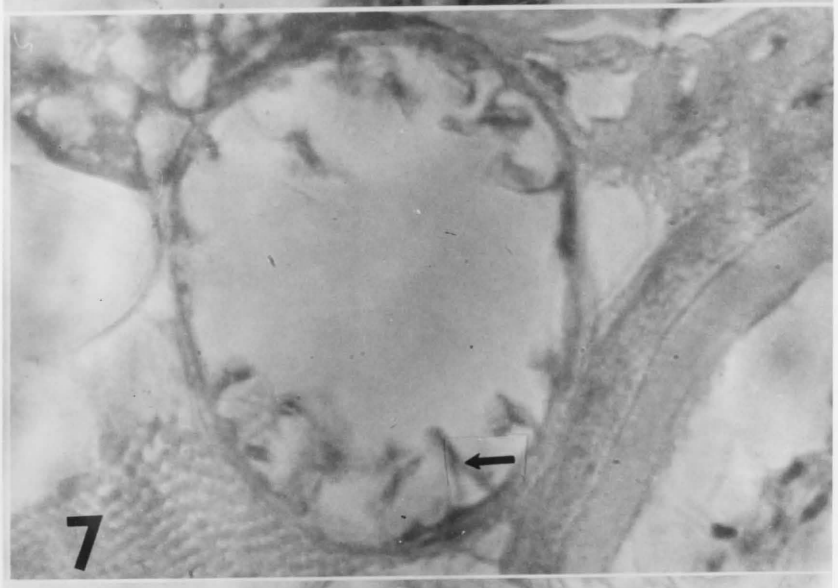

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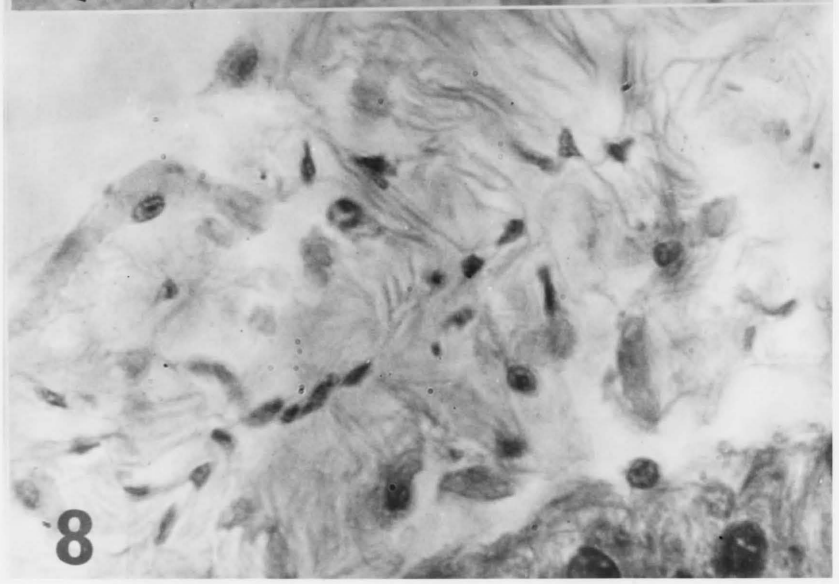

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Figure 9. Shows salivary ducts from a pair of salivary glands that join and form a salivary syringe. Note the epithelial cells (seen as dark spots) on the duct (S. rugglesi female).

Figures 10-14. Sensilla in the buccal cavity of female black-fly.

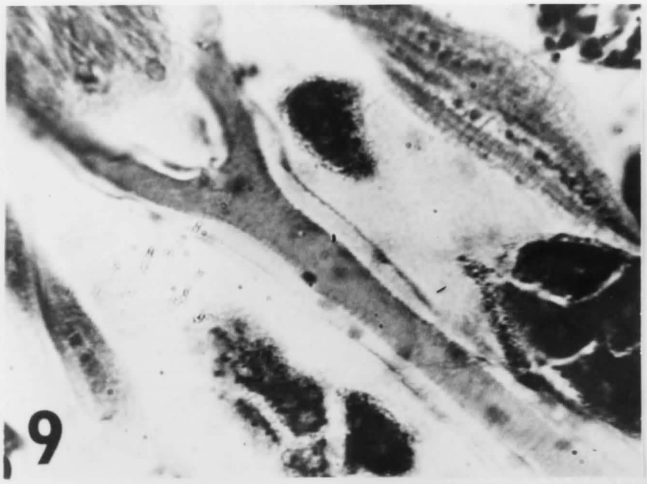
Fig. 10. Labral medial sensillum. S. quebecense female

Fig. 11. Labral lateral sensilla. P. decemarticulatum female.

Fig. 12. Sensillum of cibarial pump. P. decemarticulatum.

Fig. 13. Sensilla of cibarial pump from whole mount showing a pair. The sensilla in the inset circle are higher magnification of Fig. 13. P. fuscum.

Fig. 14. Fine dendrites (1) leading from labral lateral sensilla (2). S. quebecense female. Phase contrast.



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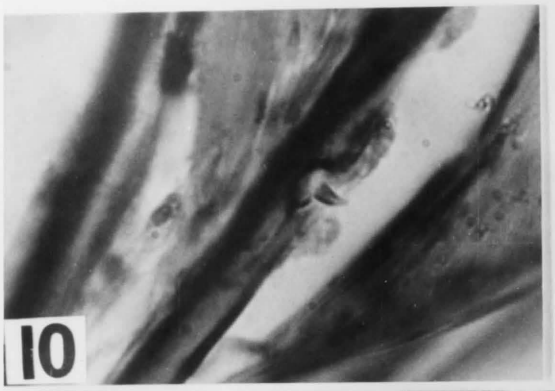

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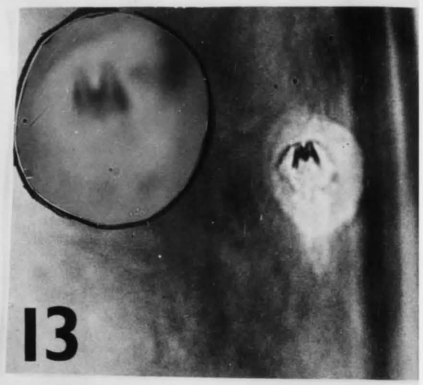

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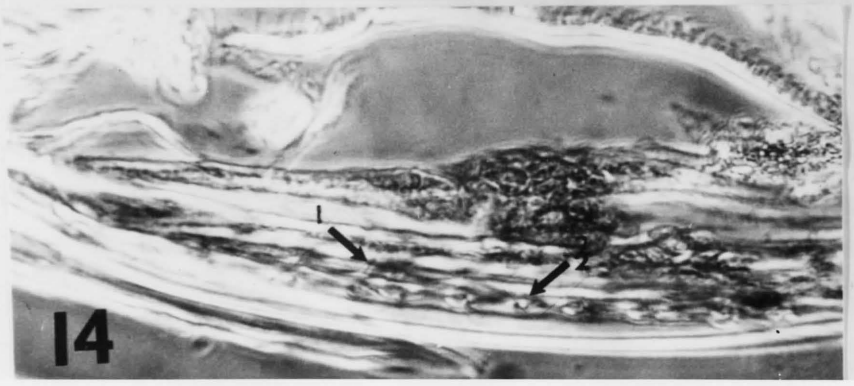

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Figures 15-17. Spines at junction of cibarial and pharyngeal pumps.

Fig. 15. S. vittatum female.

Fig. 16. S. luggeri female (1-spines, 2-cibarial sensilla).

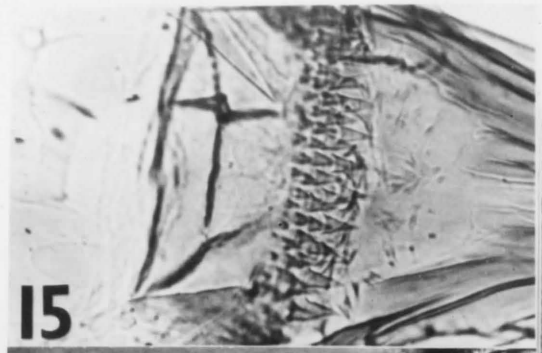
Fig. 17. S. venustum female (for spines see arrow).

Figures 18-20. Peritrophic membrane-like material, "the old gut content" in the midgut of S. decorum fed on sugar only.

Fig. 18. Membrane-like material in anterior midgut of male.

Fig. 19. A pear-shaped "old gut content" in the midgut of female.

Fig. 20. Higher magnification of Fig. 19.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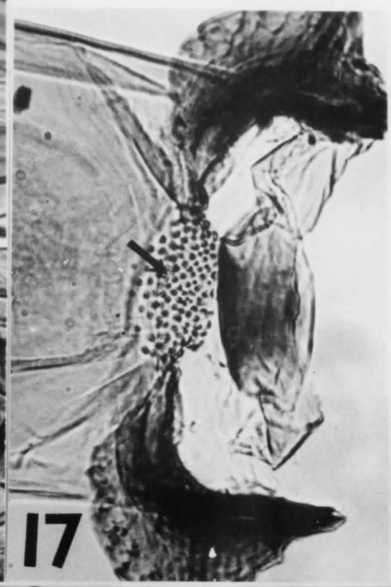
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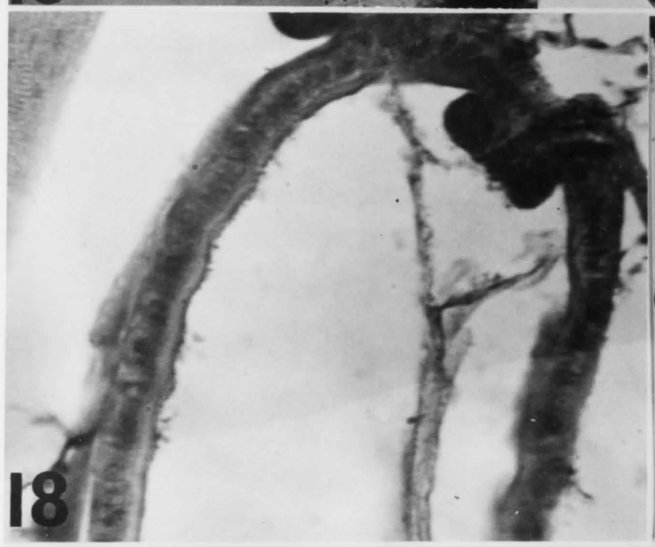
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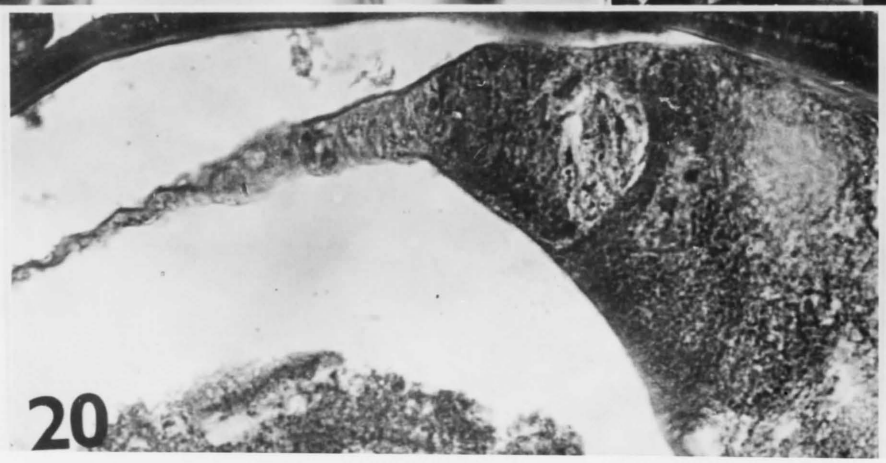
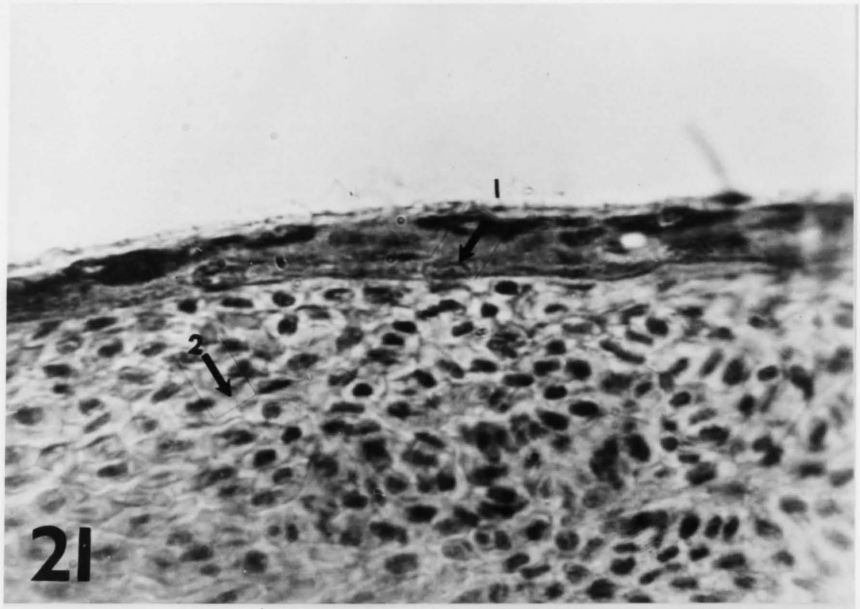

20

Figures 21-22. Longitudinal sections of posterior midgut immediately after female fed on chicken blood.

Fig. 21 shows that midgut epithelial cells (1) and blood (2) are in direct contact, showing no peritrophic membrane formed. S. decemarticulatum.

Fig. 22 shows an "old gut content" in pyloric region partly projected into the hindgut. S. croxtoni.



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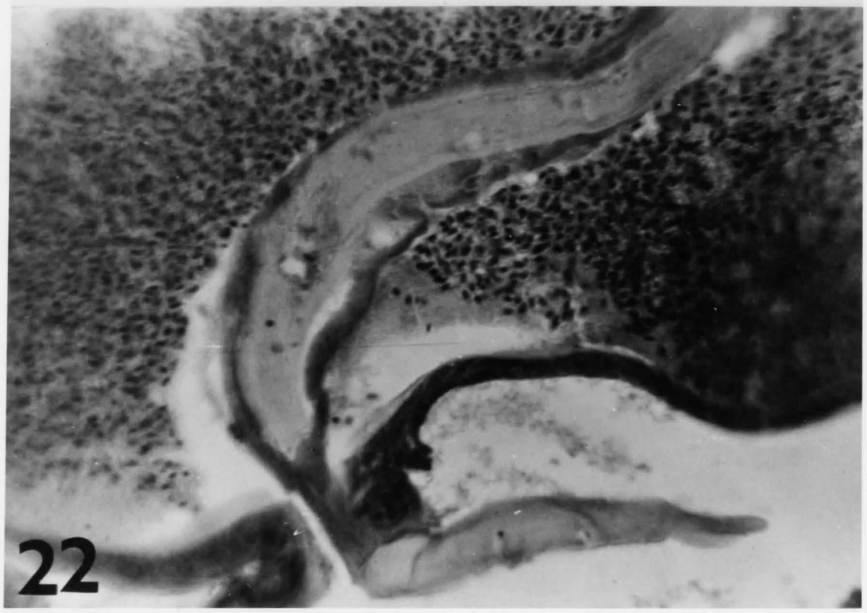


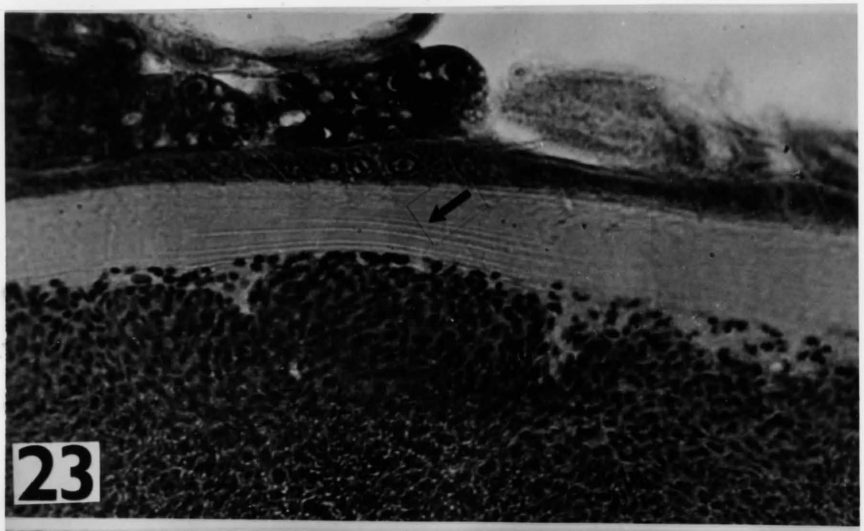
22

Figures 23-25. Peritrophic membrane in posterior midgut.

Fig. 23. Thick and viscous membraneous substance surrounds entire blood mass, sometimes showing laminated layers (arrow). S. aureum female, 3 hr after feeding on chicken blood.

Fig. 24. The other type of thicker membrane. The numbers (1, 2) indicate laminated layers. S. aureum females, 3 hr after feeding on chicken blood.

Fig. 25. The membrane solidified outwardly (arrow) and detached from midgut wall, but still firmly attached to blood mass. Note also the distinct lamination of the membrane. S. quebecense female, 6 hr after feeding on chicken blood.



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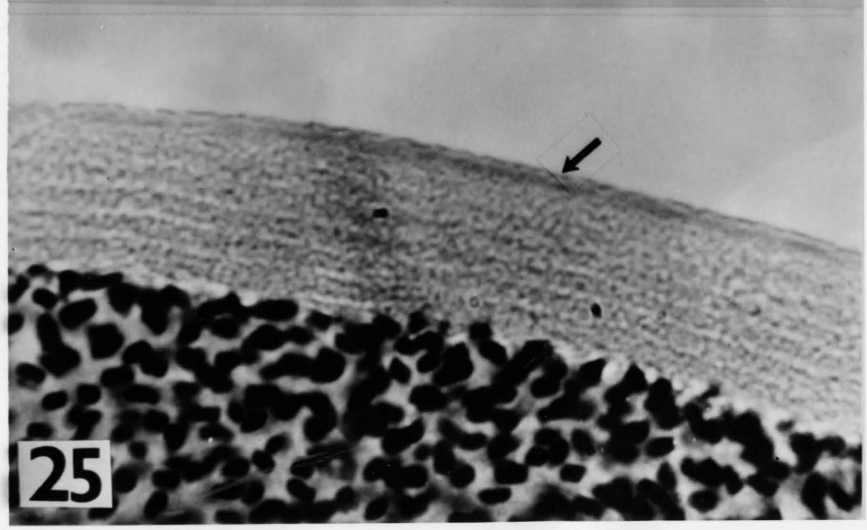

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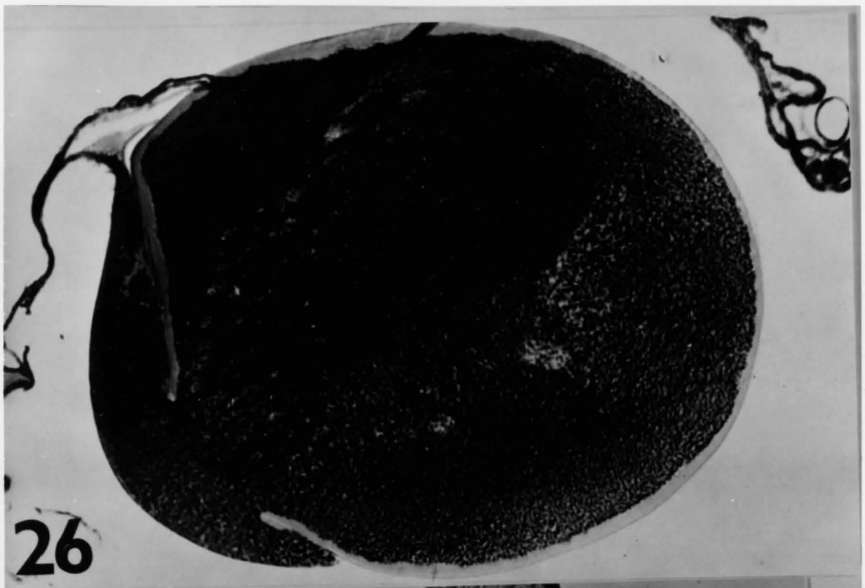
Figure 26. Peritrophic membrane near anterior end (left) of blood mass only partly surrounding the blood mass, outer portion of which is in direct contact with gut wall. S. quebecense female after feeding on chicken blood.

Figure 27. Double membrane formation (1,2) in the midgut of S. aureum female, 12 hr after feeding on chicken blood. Cross section of mid-point of posterior midgut.

Figure 28. Peritrophic membrane incomplete posteriorly, allowing outer portion of blood mass to make direct contact with the gut wall. Arrow shows the opening to the hindgut. S. quebecense female, 12 hr after feeding on chicken blood.



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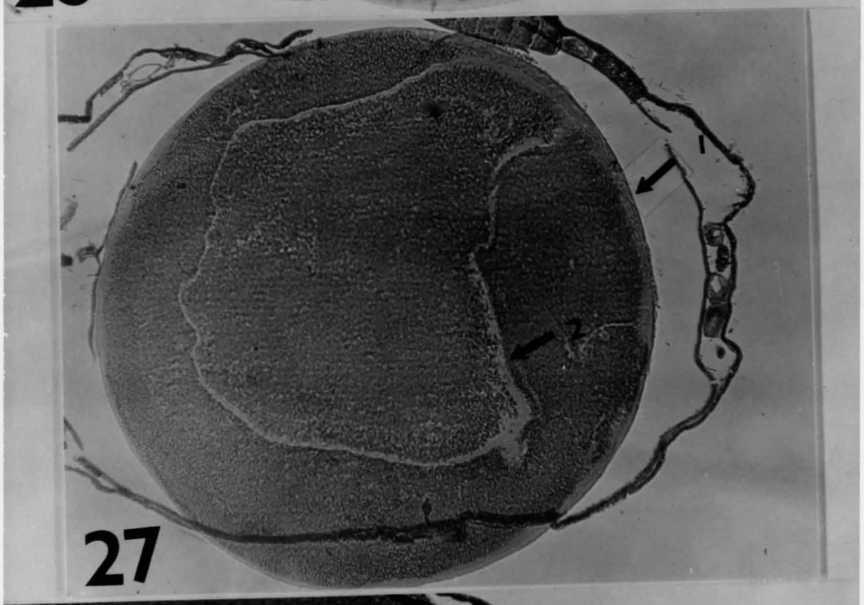

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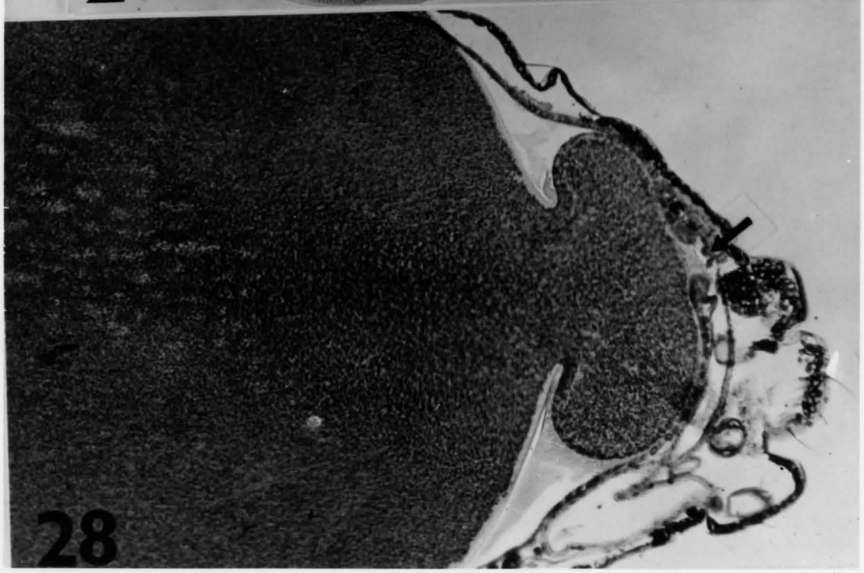

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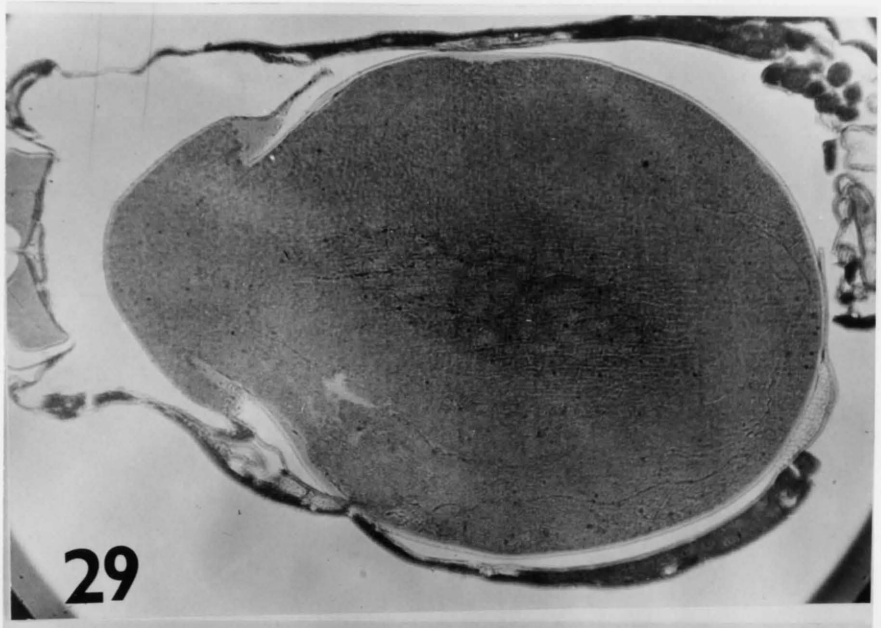
Figure 29. The constricted peritrophic membrane near anterior end of blood mass. S. quebecense female, 16-24 hr after feeding on chicken blood.

Figure 30. Thick and well solidified peritrophic membrane in pyloric region which is separated from gut wall. Note the membrane wrinkled outwardly. S. aureum female, 48 hr after feeding on chicken blood.

Figure 31. Some females showed thin peritrophic membrane partly separated from the blood mass. S. aureum female, 48 hr after feeding on chicken blood.



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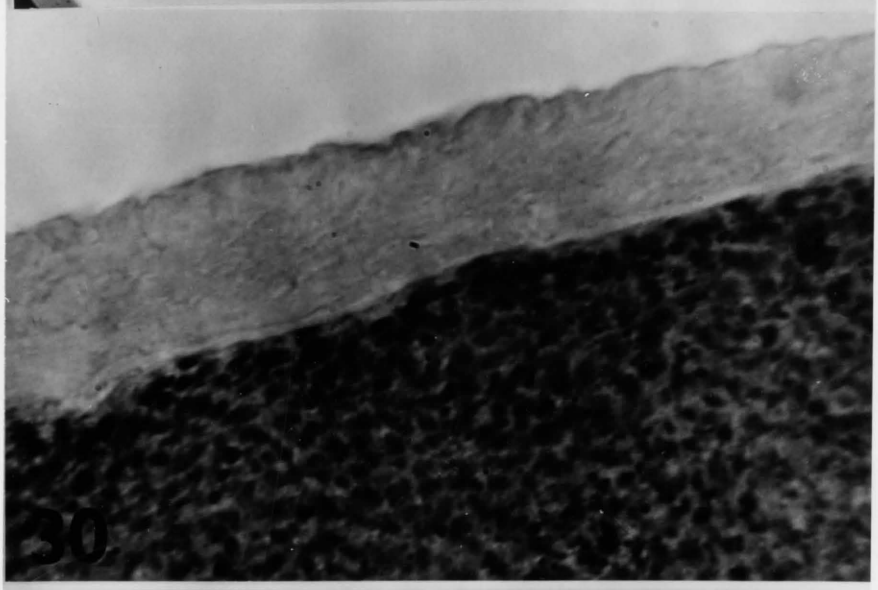

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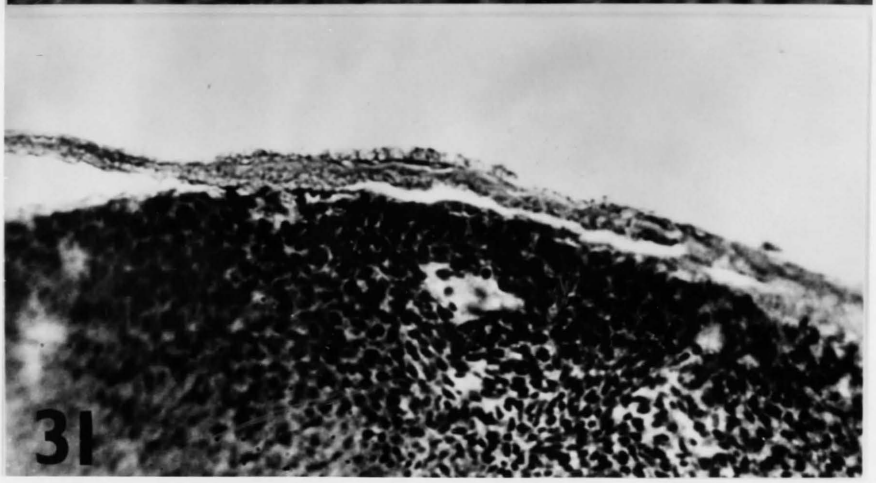

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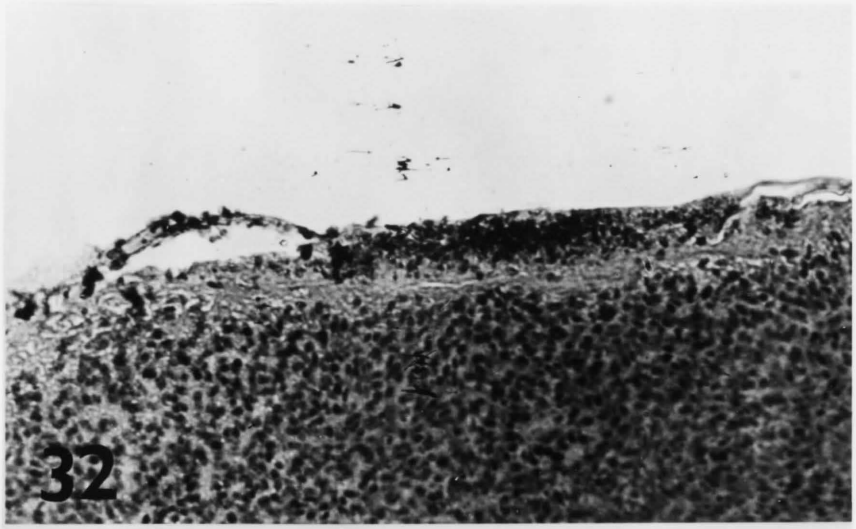
Figure 32. Peritrophic membrane incorporated with altered blood is being disintegrated. S. quebecense female, 68 hr after feeding on chicken blood.

Figure 33. Thick and wrinkled peritrophic membrane, with inclusions. S. aureum female, 72 hr after feeding on chicken blood.

Figure 34. Part of peritrophic membrane in pyloric region is digested. This will be discharged with altered blood into the hindgut. S. aureum female, 116 hr after feeding on chicken blood.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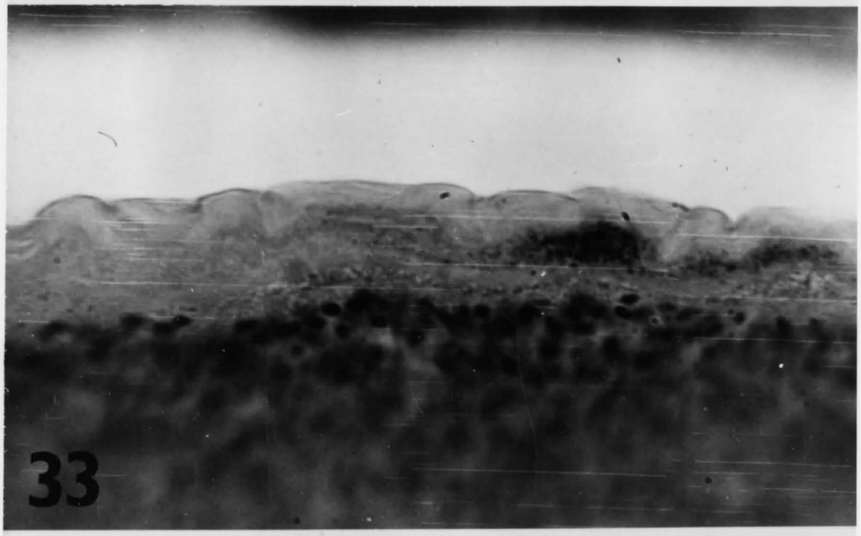
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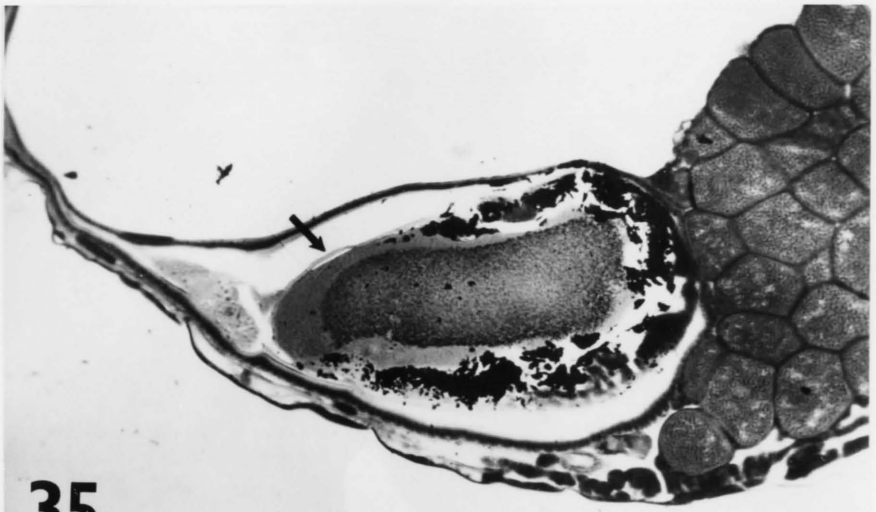
34

Figure 35. Intact peritrophic membrane (arrow) is seen around the undigested blood mass. No membrane was seen around the altered blood. S. quebecense female, 126 hr after feeding on chicken blood.

Figure 36. Advanced blood digestion (black) in pyloric region, while anterior part of blood mass is still undigested. Developing eggs to right. S. aureum female, 169 hr after feeding on chicken blood.

Figure 37. Altered blood surrounds the undigested blood in the center and the waste product is discharged into the hindgut (upper right). P. decemarticulatum female, 183 hr after feeding on chicken blood.



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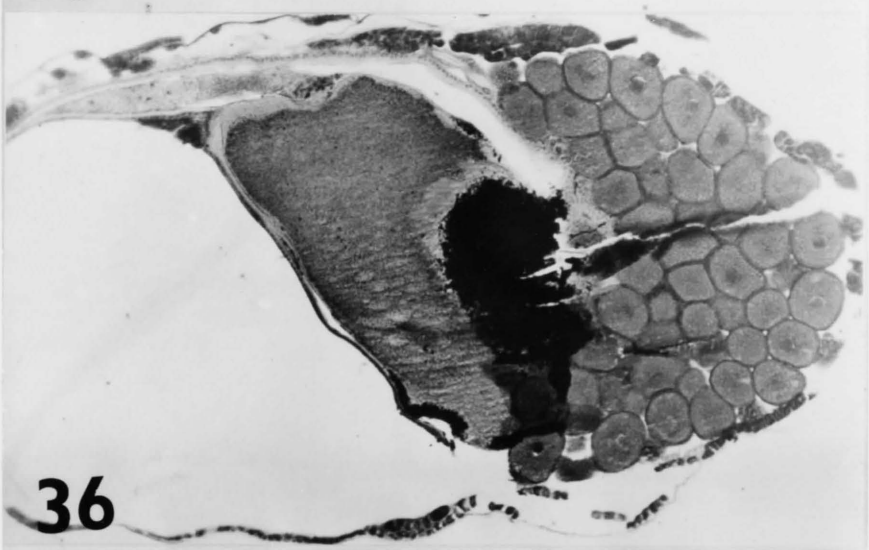

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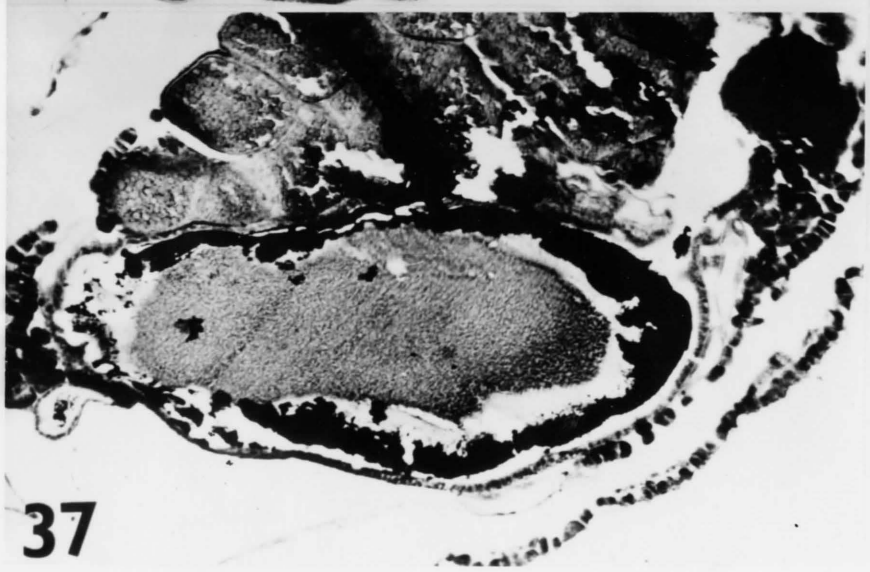

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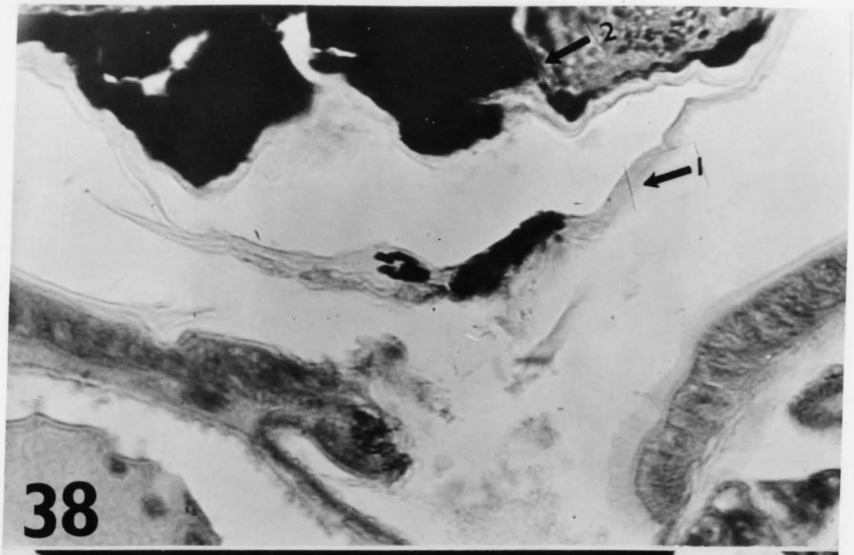
Figures 38-40. Peritrophic membrane and wast product are being discharged into the hindgut.

Fig. 38. Arrow shows disintegrating membrane. S. quebecense female, 68 hr after feeding on chicken blood.

Fig. 39. S. quebecense female, 126 hr after feeding on chicken blood.

Fig. 40. P. decemarticulatum female, 183 hr after feeding on chicken blood.



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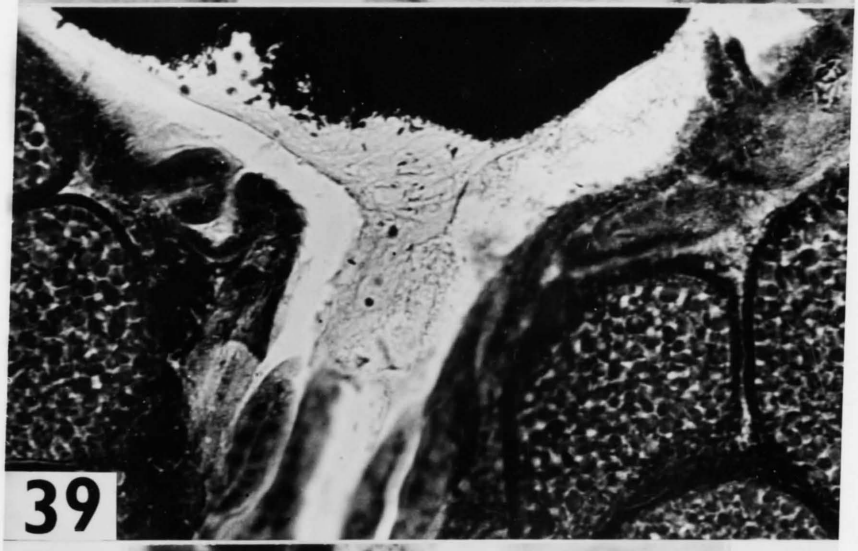

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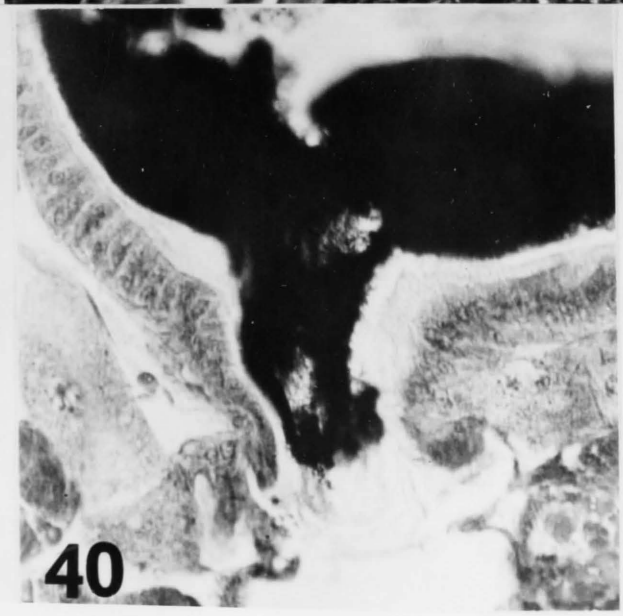


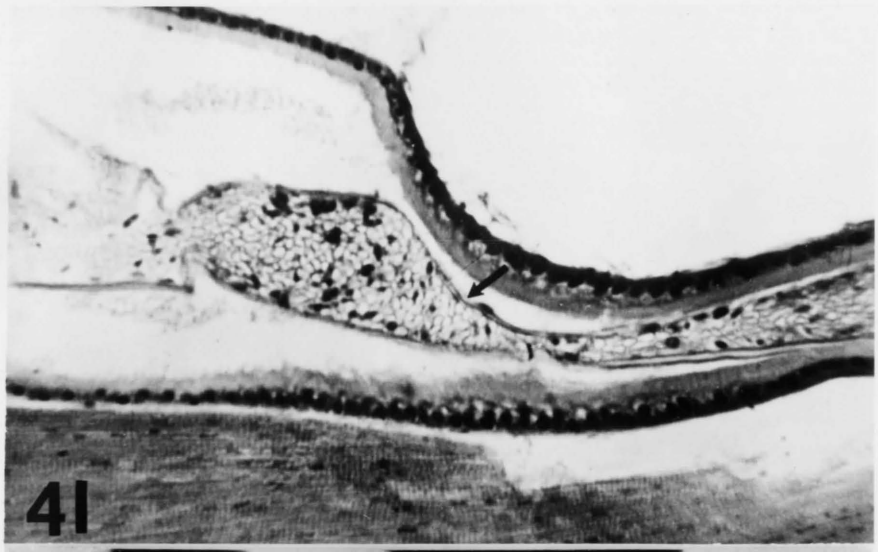
40

Figures 41-43. Peritrophic membrane in anterior midgut.

Fig. 41. Small amount of blood is surrounded by thin membrane (arrow). S. venustum female, 1 hr after feeding on moose blood.

Fig. 42. Same as Fig. 41. P. decemarticulatum female, 1 hr after feeding on chicken blood.

Fig. 43. Gelatinuous substance accumulated near front end of anterior midgut. S. aureum female, 3 hr after feeding on chicken blood.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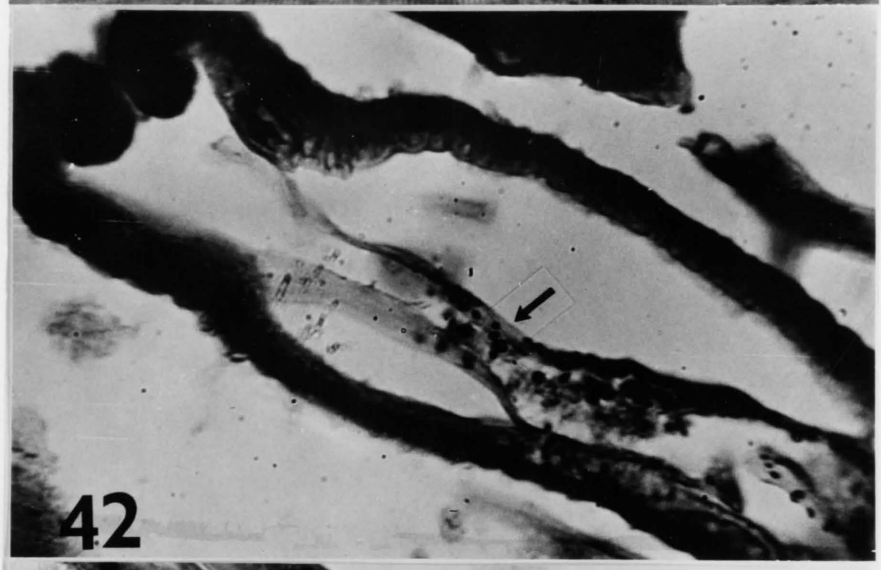
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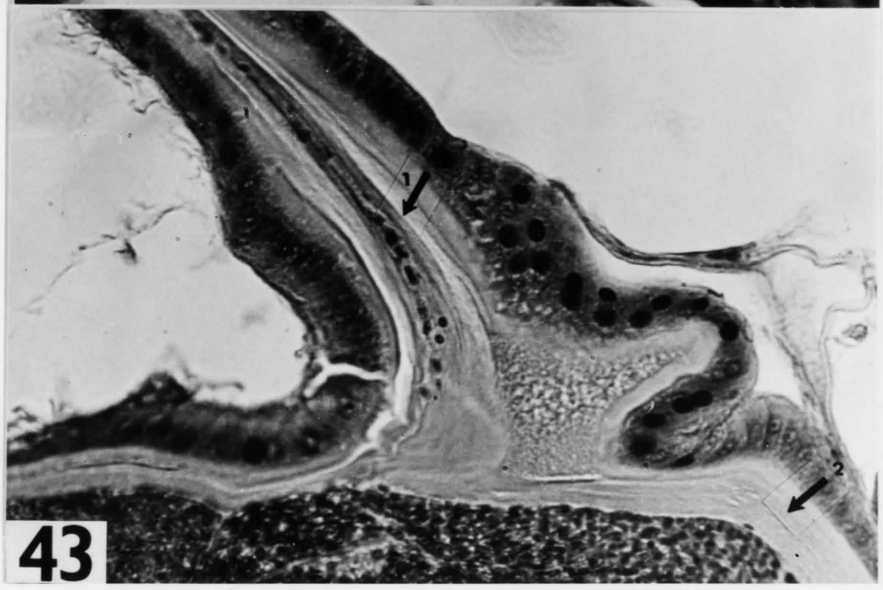
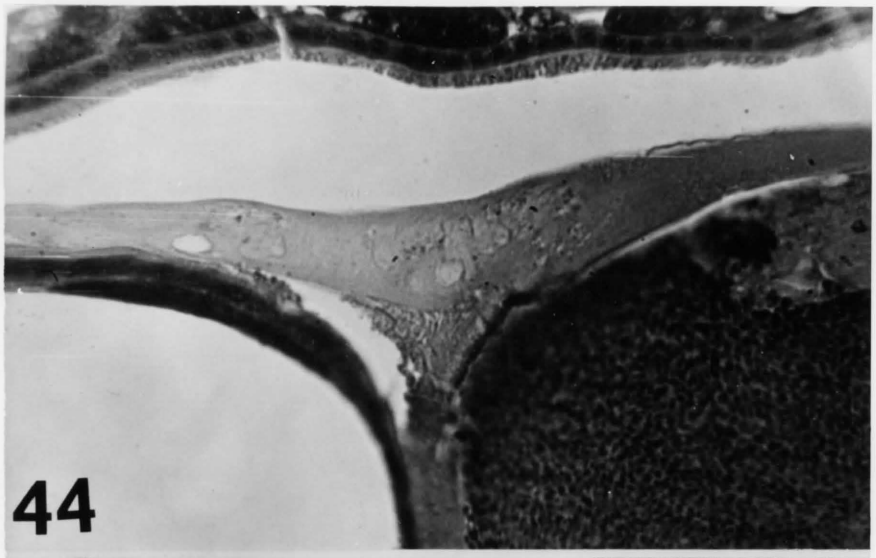

43

Figures 44-46. The accumulated material in anterior midgut.

Fig. 44. Almost solidified material. S. aureum female, 48 hr after feeding on chicken blood.

Fig. 45. Gritty particles are enclosed by a thin membrane. Note also the first membrane (arrow) which envelop the blood mass. S. quebecense female, 126 hr after feeding chicken blood.

Fig. 46. Yeast-like materials are enveloped by a thin membrane (arrow). S. aureum female, 169 hr after feeding on chicken blood.



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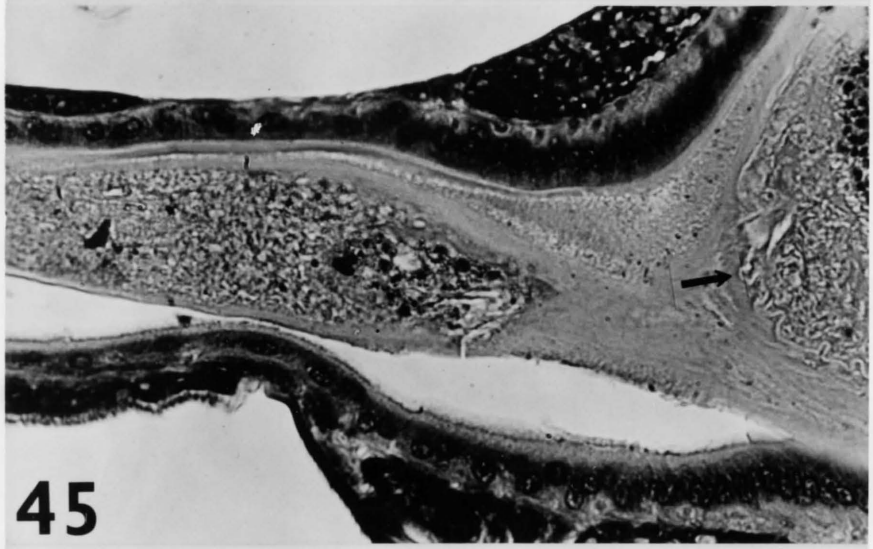

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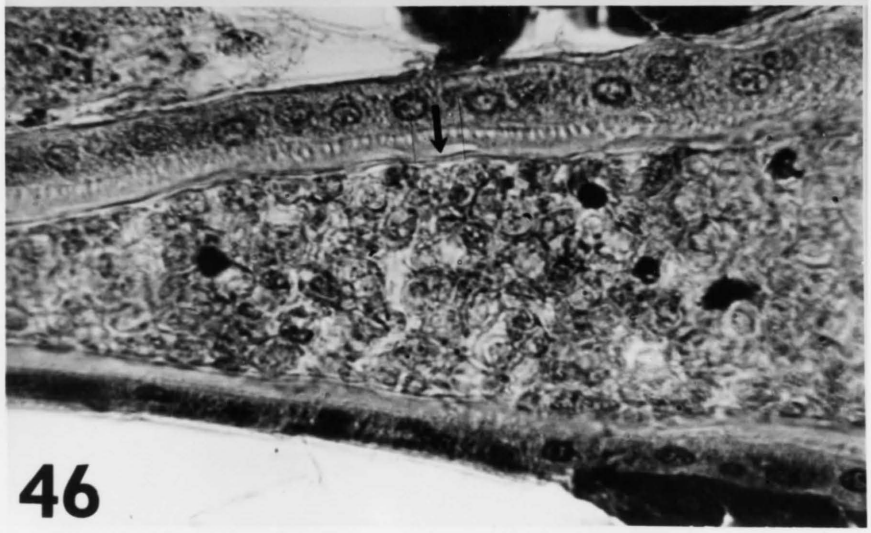

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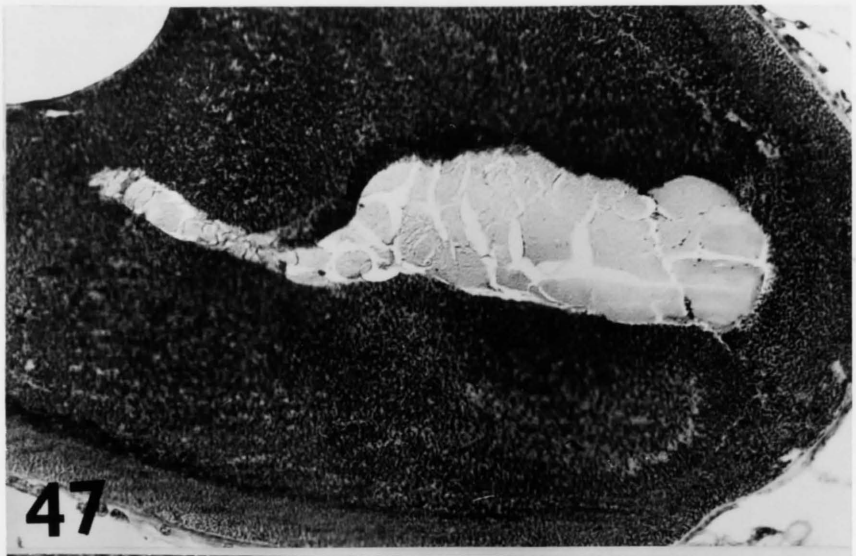
Figure 47. An "old gut content" surrounded by blood mass. The content is actually reached to pyloric region. P. decemarticulatum female, 1-2 hr after feeding on chicken blood.

Figure 48. The other type of "old gut content" which is gripped by pyloric sphincter. The content shows several portions divided by membrane. Note newly forming membrane on right hand side. P. decemarticulatum female, 1 hr after feeding on chicken blood.

Figure 49. Crop duct full of erythrocytes. S. vittatum female, 3-6 hr after a meal of erythrocyte-sucrose mixture.



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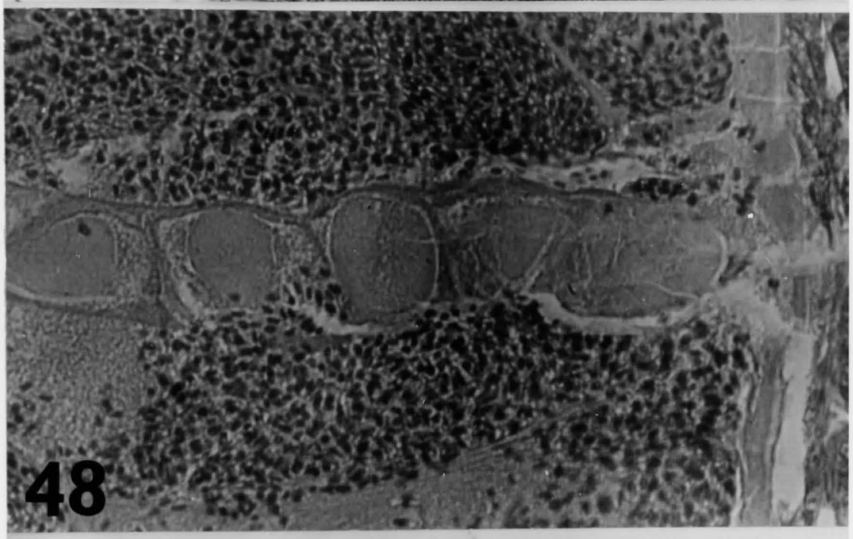

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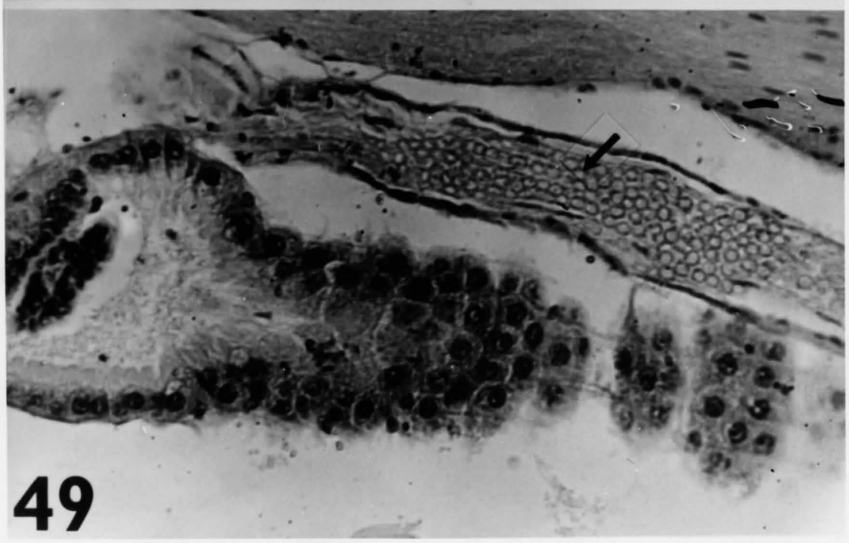
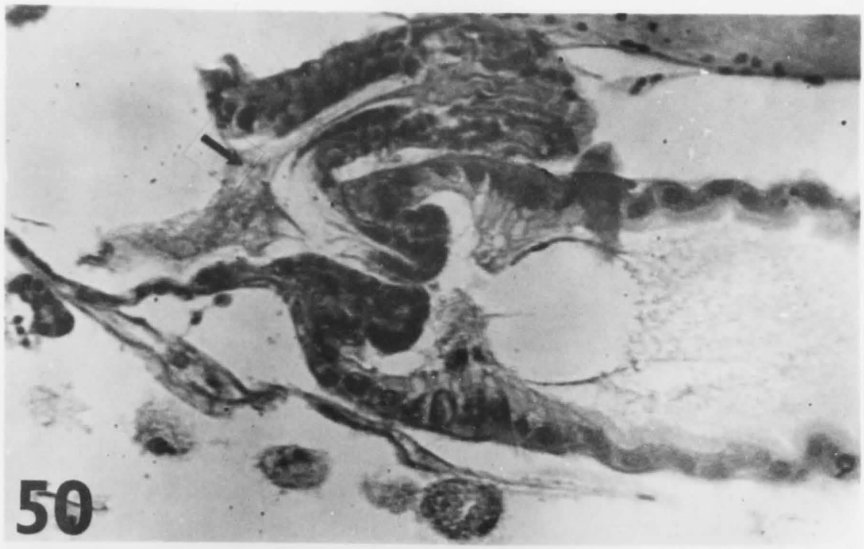


49

Figure 50. Same as Fig. 49 but further inner section, showing the erythrocyte-sucrose mixture stretched from crop duct to cardia.

Figure 51. Peritrophic membrane in pyloric region. S. venustum females, 5-12 hr after a meal of blood-sucrose mixture.

Figure 52. An "old gut content" in the midgut of S. vittatum female, 2 hr after a meal of erythrocyte-sucrose mixture. This fly emerged from pupa in the laboratory and had no previous blood meal.



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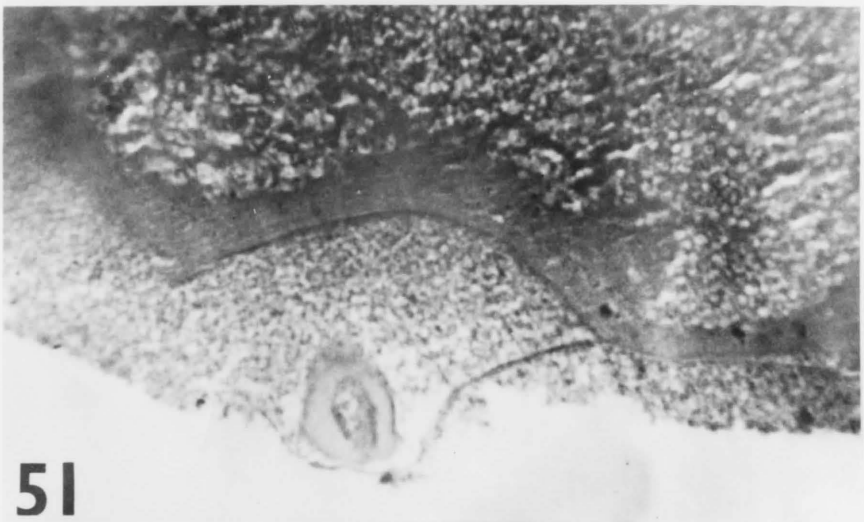

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Figure 53. Oligosaccharides (O) on chromatograms prepared with 2% sucrose incubated with homogenates of unfed S. venustum females.

1, 4, 3; whole fly homogenates

8, 18; midgut homogenates

SO; sucrose and boiled homogenate of whole fly

SFG; sucrose, glucose and fructose with boiled homogenate of whole fly

F; fructose, G; glucose, S; sucrose.

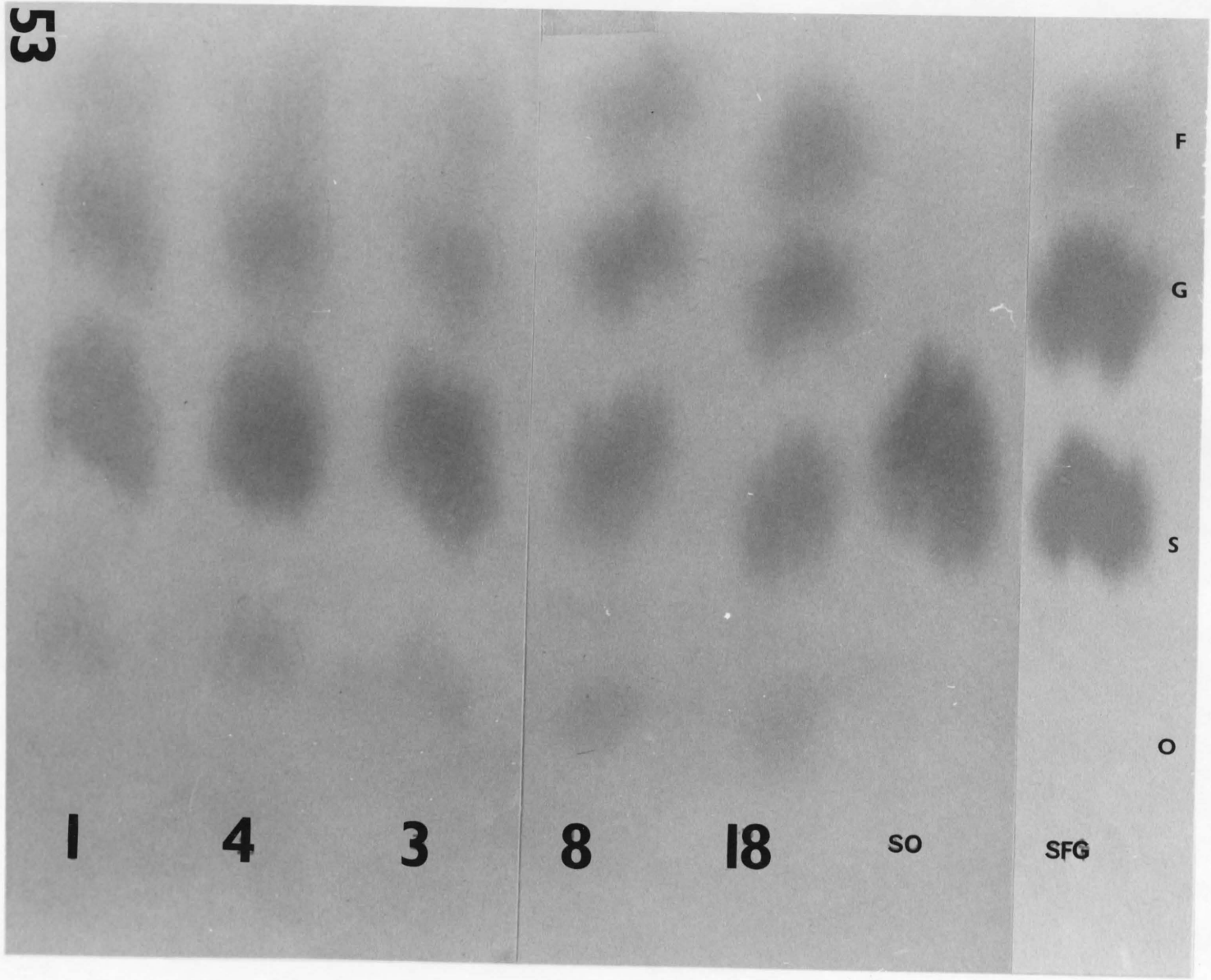

53
F
G
S
O
1
4
3
8
18
SO
SFG

TABLE 1. Details of the black-flies used for histology including species, type of food ingested, number of flies sectioned, and the various invervals after feeding when flies were fixed for sectioning.

Species	Diet[a]	No. flies	Hours following feeding												
			0	1	3	6	12	24	48	72	96	120	144	168	192
P. decemarticulatum	Chicken blood	81	x[b]	x	x	x	x	x	x	x		x	x		x
S. aureum	Chicken blood	36	x	x	x		x	x	x	x		x	x	x	
S. croxtoni	Chicken blood	15	x	x	x										
S. quebecense	Chicken blood	51	x	x		x	x	x	x	x	x	x			
S. decorum	Sugar & water	10	(5 females and 5 males)												
S. venustum	Duck blood	15	x	x	x	x	x	x							
S. venustum	Human blood	39	x		x	x	x	x							
S. venustum	Moose blood	45		x	x		x	x	x	x	x	x			
S. venustum	Deer blood	39		x	x	x		x	x	x	x	x			
S. venustum	Blood-sucrose[c]	35		x				x	x						
S. venustum	RBC-sucrose[d]	15		x				x	x						
S. vittatum	Blood-sucrose[c]	15		x				x	x						

[a]Blood meals from these hosts: humans, northern white-tailed deer, eastern moose, Pekin duck and bantam chicken.

[b]indicates sections made at the time after a blood meal.

[c]Human blood-sucrose mixture.

[d]cow erythrocytes-sucrose mixture.

TABLE 2. Measurements of the salivary glands of adult female simuliids.

Species	Age(hr)	Average size of salivary gland[a] (μ)		
		S.R.	P.A.	D.A.
S. decorum	0 - 12	141/103[b]	257/71	353/71
S. decorum	24	195/148	347/82	384/87
S. decorum	72	210/116	302/90	377/83
S. venustum	168	130/115	206/66	265/58
S. vittatum	168	185/103	296/77	289/63

[a]From 10 females (20 salivary glands).

S.R.: salivary reservoir

P.A.: proximal arm

D.A.: distal arm

[b]Length/width

TABLE 3. The percentage of unfed, partially fed and fully fed S. venustum females in relation to the proportion of blood and saturated sucrose solution in various mixtures presented as food.

Host	Blood[a] (%)	No. flies tested	Unfed(%)	Part.fed(%)	Fully fed (%)	Food in gut/crop[b]
Human	100	31	64.5±17.6	35.5±17.6	0	5/0
	90	45	33.3±14.0	66.7±14.0	0	0/5
	80	68	8.8± 6.8	91.2± 6.8	0	0/5
	70	67	1.5± 2.8	97.0± 2.8	1.5±2.8	0/5
	60	64	0	20.0±10.0	80.0±10.0	0/5
	50	51	0	9.8± 8.2	90.2± 8.2	0/5
Chicken	100	41	100	0	0	-
	80	54	100	0	0	-
	50	53	9.4± 8.4	77.4± 8.4	13.2± 8.4	0/5
Duck	100	52	98.0± 3.8	2.0± 3.8	0	-
	80	55	30.9± 7.8	69.1± 7.8	0	0/5
	50	58	5.2± 5.2	86.2± 5.2	8.6± 5.2	0/5

[a]Blood was diluted in saturated sucrose solution.

[b]Five flies of each group dissected to note disposition of meal.

TABLE 4. Distribution of different blood-sucrose mixtures in midgut and crop of S. venustum females.

Sucrose (moles) in whole blood[a]	No. females exposed	No. of females having the mixture in		
		Crop	Crop-midgut	Midgut
0.5	25	5	10	0
0.4	25	1	18	1
0.3	25	1	11	2
0.2	25	0	2	1
0.1	25	0	4	5
0.05	25	0	2	3
0.02 - 0.01	50	0	0	4

[a]Whole blood was diluted with 1 M sucrose solution.

TABLE 5. Volume of crop content and number of erythrocytes in the crop of S. venustum female after feeding on different mixtures of human erythrocytes and sucrose solution.

RBC : Sucrose	No.RBC/µl mixture x 1000	No. tests	Midgut	Crop	Volume of crop content (µl)	No.RBC/µl in crop x 1000
4 : 1[a]	555	1	slight	full	1.30	45
		2	trace	partial	0.85	45
		3	trace	partial	0.80	45
		4	slight	partial	0.81	54
2 : 1	417	1	trace	partial	0.85	20
		2	trace	partial	0.85	21
		3	trace	slight	0.45	45
1 : 1	306	1	trace	slight	0.60	36
		2	trace	slight	0.50	45
		3	trace	slight	0.45	44
1 : 2	159	1	trace	slight	0.45	29
		2	trace	slight	0.65	18
		3	trace	slight	0.55	47
1 : 4	58	1	trace	full	1.35	34
		2	trace	full	1.30	43
		3	trace	full	1.25	36

[a]Four parts 80% erythrocytes and one part 1 M sucrose solution.

TABLE 6. Agglutinin activity in the salivary gland extracts of adult female simuliids[a].

Species	Erythrocytes from			
	Human	Cow	Duck	Chicken
S. decorum	11/15[b]	0/10	5/7	0/7
S. venustum	15/15	0/10	12/13	10/10
S. vittatum	9/10	-	10/10	-

[a]Five to 15 days old.

[b]No positive/no. tests

TABLE 7. Anticoagulant activity in the salivary gland extracts of adult female simuliids[a].

Species	No. tests	Recalcification time (min)	
		Individual readings	Mean
Control	12	3 5 4 3 4 4 4 3 4 5 5 5	4.2
S. decorum	10	10 6 8 10 8 12 13 14 13 13	10.7
S. venustum	11	8 8 10 11 25 40 8 40 8 10 13	16.5
S. vittatum	4	8 6 15 9	9.5

[a]one to two weeks old

TABLE 8. Trypsin activity (μg/ml) in various tissue extracts of S. venustum females[a] fed dry sucrose and water.

No. tests	Tissues analysed[b]					
	Midgut	M.T.	S.G.	R.F.	Carcass	W.F.
1	2.9	0	0	2.1	0.1	4.6
2	3.7	0	0	1.4	0	4.5
3	3.0	0	0	1.6	0.2	5.0
4	3.2	0	-	1.5	0	4.9
Mean	3.20	-	-	1.65	-	4.75

[a]Females collected in September.

[b]M.T.: Malpighian tubule

S.G.: salivary gland

R.F.: residual fluid

W.F.: whole fly

TABLE 9. Trypsin activity (μg/ml) in various tissue extracts of S. venustum females[a] fed a blood-sucrose mixture (3 : 1 ratio).

Time after meal (hr)	Tissues analysed			
	Midgut	Crop	Carcass	Whole fly
6	5.7[b]	0.1	0.5	-
9	8.4	0.1	0	-
12	7.7	0	0.2	-
28	9.8	0	0	10.0
48	8.3	0.1	0	9.0
70	-	-	-	8.0
96	-	-	-	10.1
123	-	-	-	7.4

[a]Females collected in September.

[b]Single test in duplicate.

TABLE 10. Tryspin activity (µg/ml) in various tissue extracts of S. vittatum females[a] fed dry sucrose and water.

No. tests	Tissues analysed[b]				
	Midgut	S.G.	R.F.	Carcass	W.F.
1	1.2	0	0.5	0	2.2
2	2.1	0	0	0	2.0
3	1.9	-	0.8	0	1.4
Mean	1.7	-	0.4	-	1.9

[a]Females collected in September.

[b]For details, see Table 8.

TABLE 11. Trypsin activity in males and females of simuliid species.

Species	Average trypsin activity, µg/ml	
	Male	Female
P. decemarticulatum	1.2(3)[a]	1.0(5)
P. fuscum	0.5(5)	0.9(5)
C. dacotensis	1.8(3)	2.1(5)
S. venustum	3.3(5)	3.8(5)
S. vittatum	-	1.9(3)

[a]Number tests in parenthesis; each test in duplicate.

TABLE 12. Trypsin activity (μg/ml) in the homogenates of adult female simuliids after feeding on hosts.

Time after blood meal (hr)	Specimens of simuliids and hosts			
	P.decemarticulatum	S. quebecense	S. rugglesi	S. venustum
	Chicken	Chicken	Duck	Human
0 - 0.5	1.0[a]	-	3.0	3.9
1	-	7.4	4.9	4.1
3	8.5	-	5.5	7.9
6	-	-	7.6	-
9	-	-	8.2	-
12	6.3	-	8.8	-
18	5.8	-	13.5	-
24	8.6	-	13.1	-
73	8.3	16.9	-	-

[a]Single test in duplicate.

TABLE 13. Trypsin activity in S. venustum females after feeding on whole duck blood or erythrocytes.

Time after meal (hr)	Trypsin activity, μg/ml	
	Erythrocytes[a]	Whole Blood[a]
24	7.8[b]	8.9
70	8.8	9.2
92	6.5	8.8
120	8.6	10.5

[a]Eighty percent(v/v) whole blood or erythrocytes suspended in 1 M sucrose solution.

[b]Single test in duplicate.

TABLE 14. Trypsin activity in S. venustum females after feeding on whole cow blood or erythrocytes.

Time after meal (hr)	Trypsin activity, μg/ml	
	Erythrocytes[a]	Whole blood[a]
24	7.0[b]	-
36	7.2	6.8
48	6.8	-
72	-	9.6
96	-	7.8
120	8.2	7.4
168	-	7.8
194	-	8.7

[a]Eighty percent (v/v) whole blood or erythrocytes suspended in 1 M sucrose solution.

[b]Single test in duplicate.

TABLE 15. Pepsin-like activity (expressed as optical density) in homogenates of P. fuscum with and without crystallized pepsin.

Enzyme source + cryst. pepsin (homogenate)	(µg/ml)	Optical density[a]
Female	0	0
Female	0	0
Male	0	-0.01
Male	0	0
Female	50	0.283
Female	10	0.048
Female	5	0.030
Female	1	0.007

[a]Averages of 3 tests; each test in duplicate.

TABLE 16. Amylase activity[a] in homogenates of P. decemarticulatum and S. venustum incubated with or without bacteriostat.

Homogenates[c]	Number tests			
	With toluene[b]		Without toluene	
	1	2	1	2
1	0.28	0.27	0.35	0.31
2	0.30	0.29	0.34	0.32
3	0.15	0.15	0.18	0.20
4	0.10	0.09	0.14	0.13

[a]Colorimetric assay using homogenates of Eq. 3 flies/ml.

[b]0.2 ml toluene per ml reaction tube.

[c]Homogenate 1; S. venustum female, 12 hr old

Homogenate 2; S. venustum female, 24 hr old

Homogenate 3; S. venustum male, 24 hr old

Homogenate 4; P. decemarticulatum, male, 24 hr old

TABLE 17. Amylase activity[a] in various tissues of black-fly and mosquito females.

Tissues assayed	Simuliidae		Culicidae	
	S.venustum	S.vittatum	C.pipiens	A.aegypti
Whole body	0.82 (2)[b]	0.43 (1)	0.47 (2)	0.07 (2)
Midgut	0.11 (3)	0.04 (3)	0.07 (3)	0.03 (3)
Carcass	0.19 (3)	0.15 (3[c])	0.01 (3)	0.03 (3)
Carcass wash	0.24 (3)	-	0.22 (3)	0.03 (3)
Residual fluid	0.22 (3)	0.15 (3)	0.29 (3)	0.05 (3)

[a]Colorimetric assay using 10 flies, tissues or fluids/ml.

[b]Averages of number of tests in parenthesis, each test in duplicate.

[c]Not washed.

TABLE 18. Amylase activity[a] in homogenates of S. venustum females[b] at intervals after emergence from pupae.

Age (hr)	1	3	6	9	12	18	24
Optical density	0.72	0.78	0.74	1.38	1.38	1.25	1.44

[a]Colorimetric assay using 10 whole flies per ml; averages of two tests.

[b]Sugar and water were provided during the test period.

TABLE 19. Amylase activity[a] in homogenates of A. aegypti females at intervals after feeding on blood, starch or sucrose.

Meal[b]	Before meal	Time (hour) after meal						
		0	2	6	18	29	50	72
Blood	0.05	0.18	0.15	0.12	0.07	0.03	0.05	0.03
Starch	0.06	-	-	-	-	0.08[c]	0.07	0.08
Sucrose	0.05	-	-	-	-	0.05[c]	0.06	0.08

[a]Colorimetric assay using 5 mosquitoes/ml; averages of 2 tests.

[b]Fed on human blood

Ten percent starch in 10% sucrose solution

Ten percent sucrose solution

[c]Starch or sucrose was provided for 72 hours, during which the tests were made.

TABLE 20. Amylase activity[a] in homogenates of A. aegypti, immediately after feeding a human and in the host's blood.

No. test	Optical density in	
	Mosquito	Blood
1	0.40	0.45
2	0.45	0.51
3	0.43	0.50

[a]Colorimetric assay using 10 mosquitoes/ml or 26 µl blood/ml. Each test in duplicate.

TABLE 21. Invertase activity in various tissue extracts of S. venustum and P. fuscum females.

Tissues assayed[a]	Average µM sucrose hydrolysed/min/ml$_{x100}$	
	S. venustum	P. fuscum
Midgut	8.25(3)[b]	7.55(2)
Salivary gland	>0.01(2)	-0.02(2)
Crop	>0.01(5)	0.05(2)
Ovary	-	0.55(2)
Carcass	1.05(3)	2.85(2)
Carcass wash	1.46(3)	2.75(2)
Residual fluid	3.20(3)	1.55(2)

[a]Five tissues per ml.

[b]Number of tests in parenthesis; each test in duplicate.

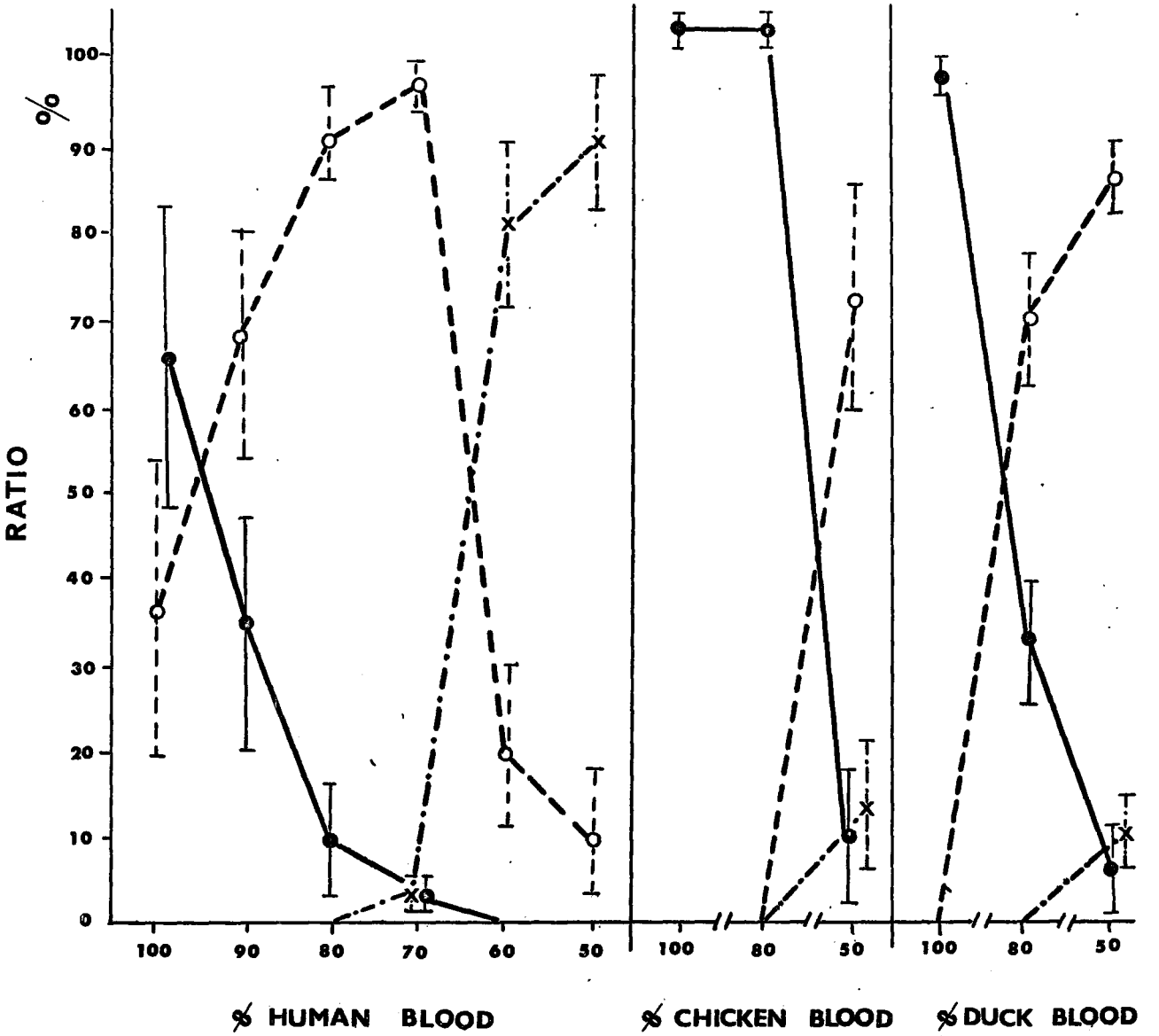
TABLE 22. Invertase activity in water-fed or sugar-fed flies of S. venustum.

Number tests[a]	µM sucrose hydrolysed/min/fly x100			
	Females		Males	
	Water-fed	Sugar-fed	Water-fed	Sugar-fed
1	4.3	6.1	3.5	4.5
2	3.4	5.2	3.9	3.3
3	3.8	3.6	4.0	3.2
4	3.3	4.5	3.5	3.3
5	4.7	5.9	4.4	
6	3.0	3.9		
7	5.5	6.0		
8	3.7			
Mean $\pm$ 1 S.D.	3.96 ± 0.59	5.03 ± 0.95	3.86 ± 0.33	3.58 ± 0.50

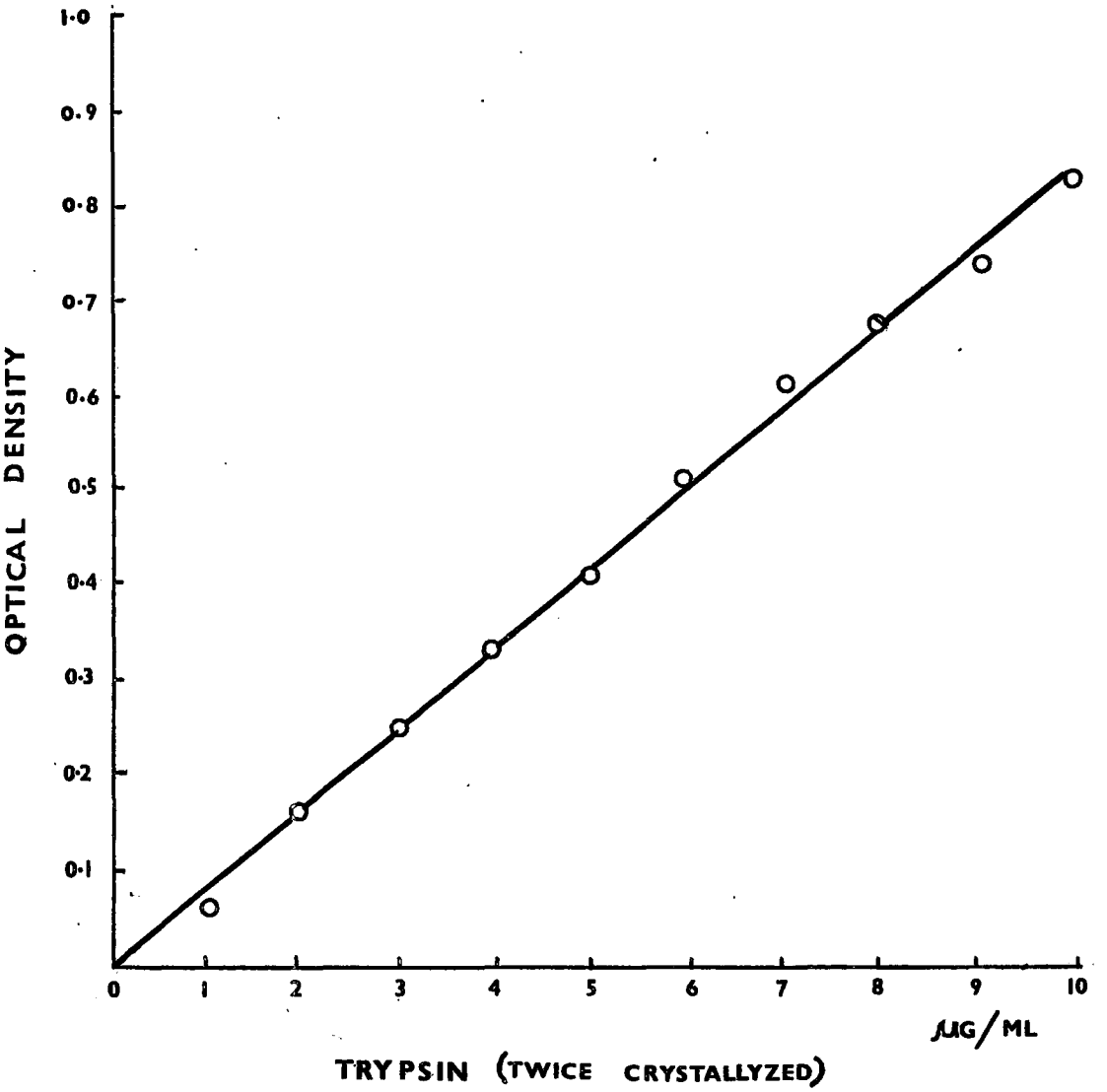
TABLE 23. Invertase activity in S. venustum females[a] at intervals after feeding on human blood.

Time after a meal (hr)	µM sucrose hydrolysed/min/fly x100			
	Number tests			Mean ± 1S.D.
	1	2	3	
0	11.0	9.3	8.8	9.7 ± 0.94
1	9.4	7.1	8.6	8.4 ± 0.60
6	11.1	7.4	9.6	9.4 ± 1.30
14	6.2	7.1	6.2	6.5 ± 0.42
24	9.5	6.9	8.9	8.4 ± 1.32
48	9.4	8.0	7.9	8.4 ± 1.01

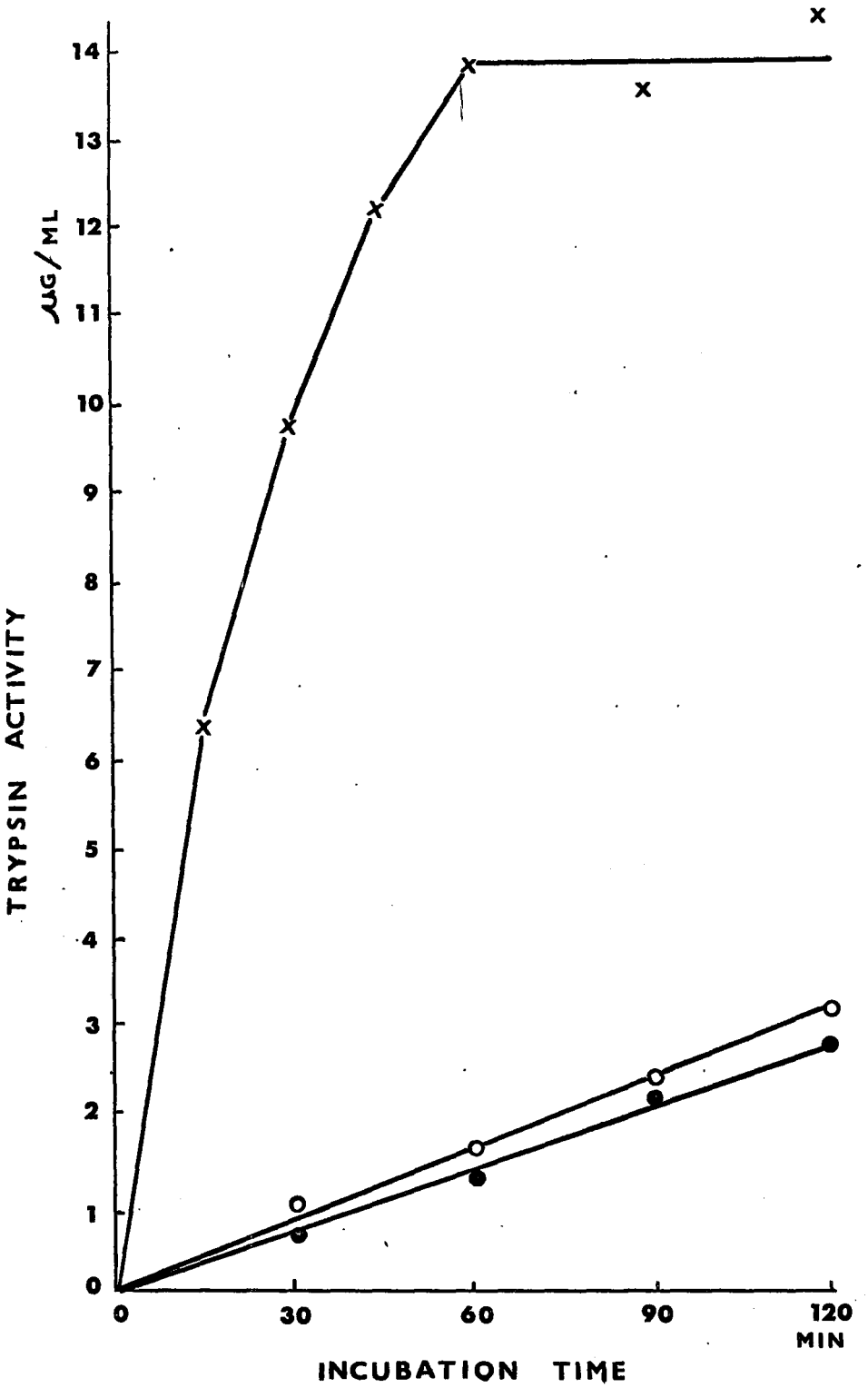
[a]Flies were kept at 18°C - 20°C.



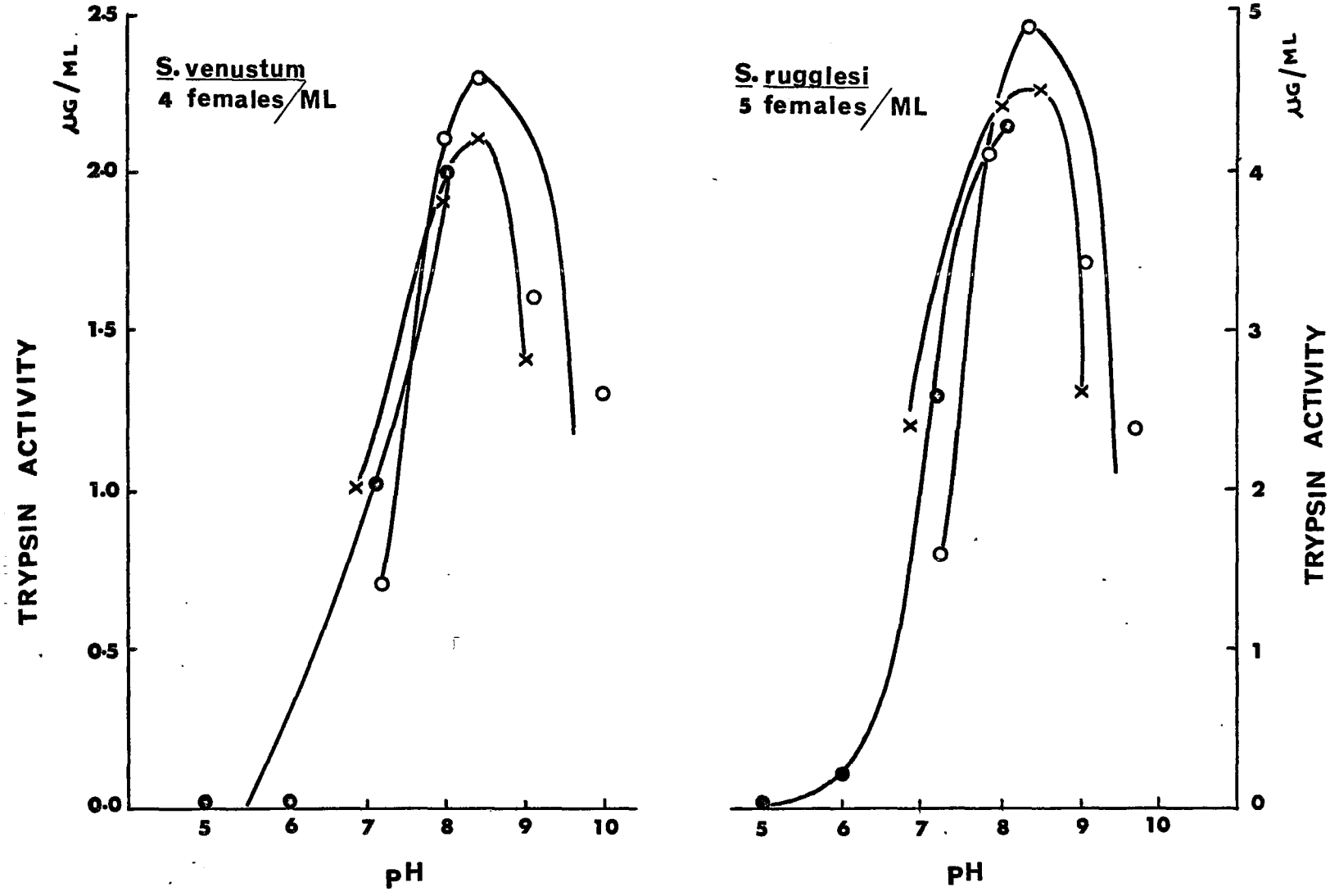
GRAPH 1. The percentage of unfed, partially fed and fully fed S. venustum females in relation to the proportion of blood and saturated sucrose solution in various mixtures presented as food. Confidence limits of the percentages taken at 0.05 x 2 level of significance. Unfed (●-●-●), partially fed (o-o-o) and fully fed (x-·-x-·-x).



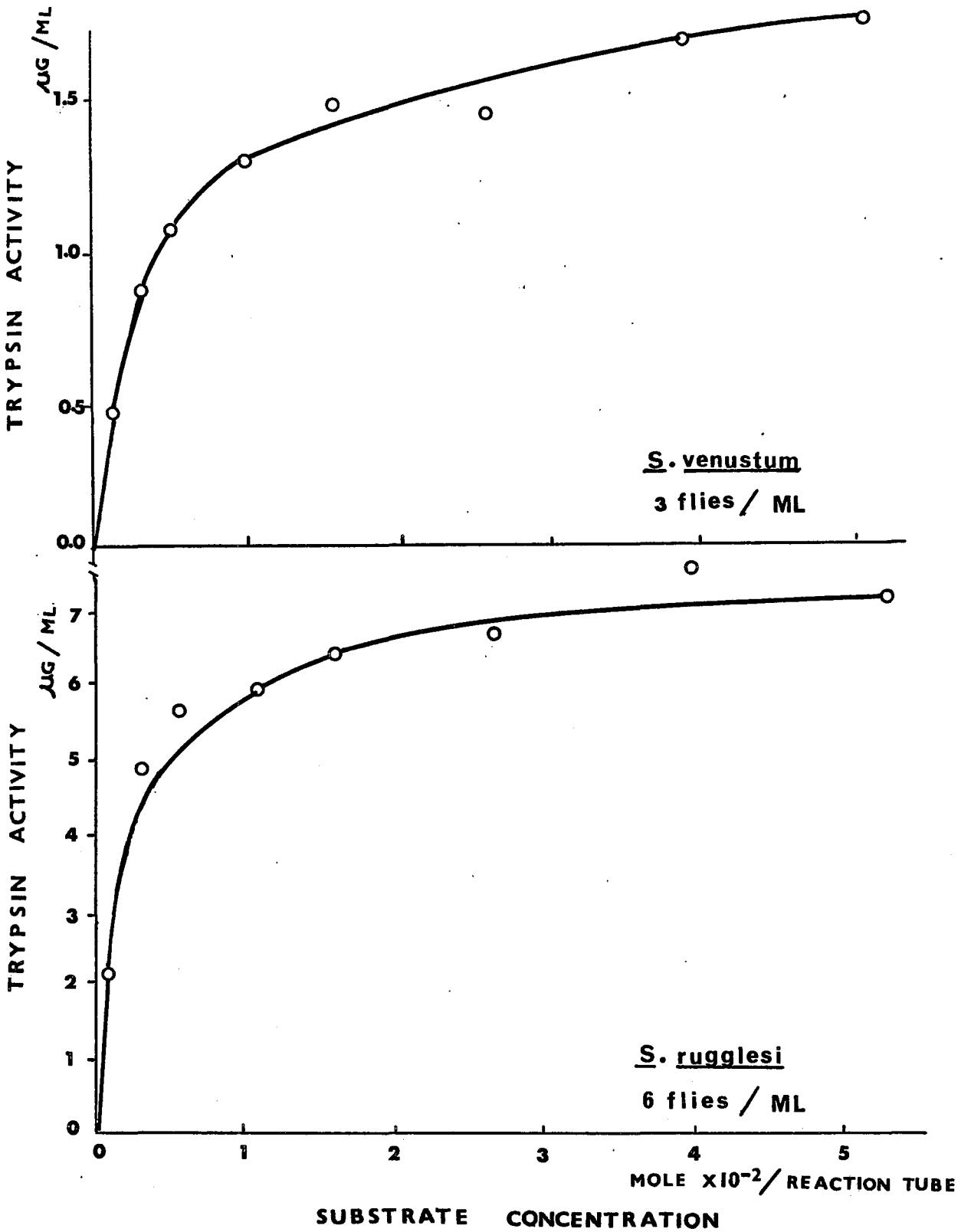
GRAPH 2. Standard curve for trypsin activity, incubated for one hour at 37°C with TAME substrate.



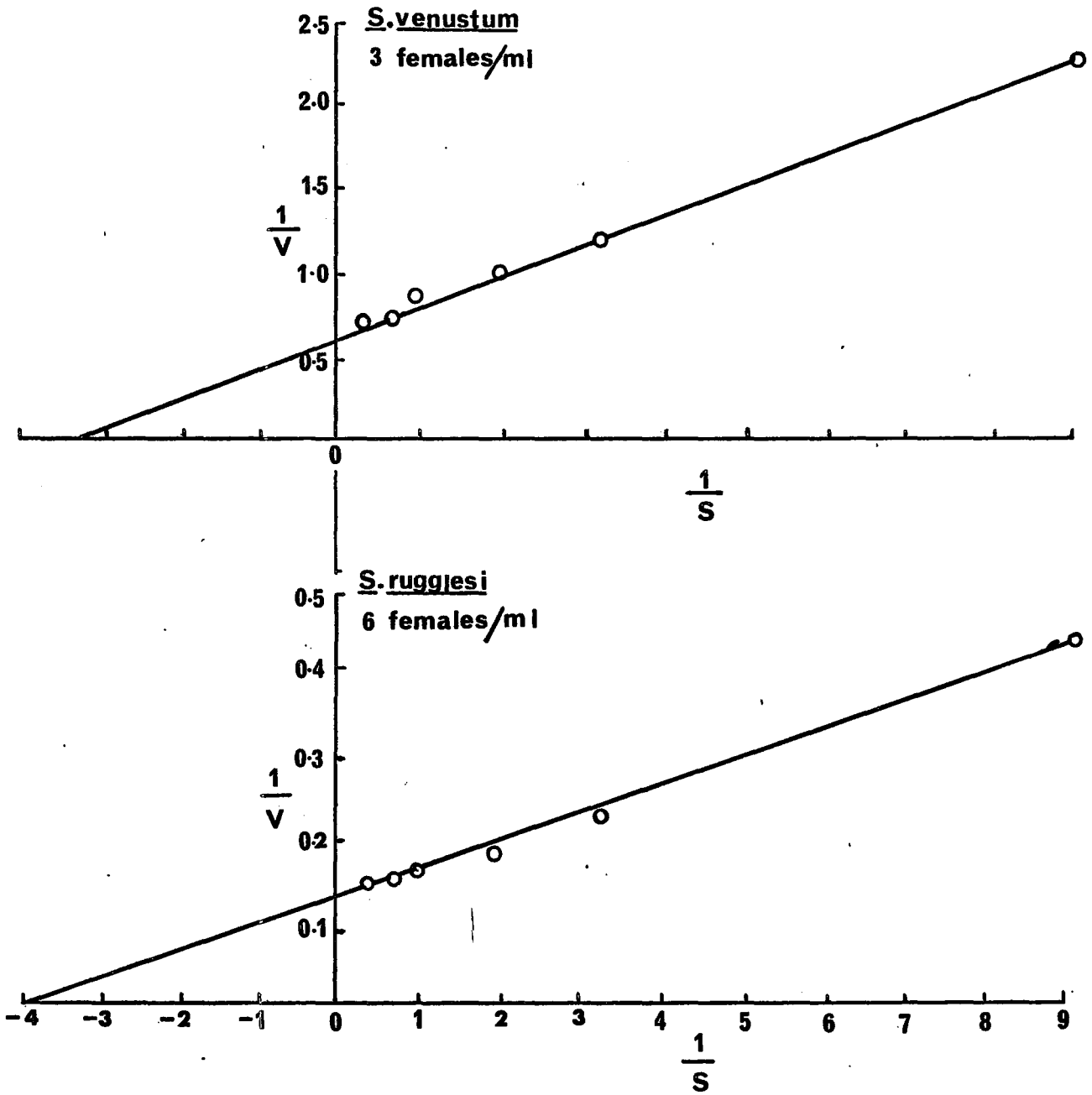
GRAPH 3. Relationship between trypsin activity of female black-fly homogenates and incubation time at 37°C. Symbols: x-x; P. decemarticulatum, 5 females/ml, •-•; P. decemarticulatum, 0.5 female/ml, o-o; S. rugglesi, 1 female/ml.



GRAPH 4. The effect of pH upon the trypsin activity of S. venustum and S. rugglesi homogenates. Buffers and symbols: •-•; Citric acid/HOP_4, o-o; Tris aminomethane/HCl, x-x; Boric



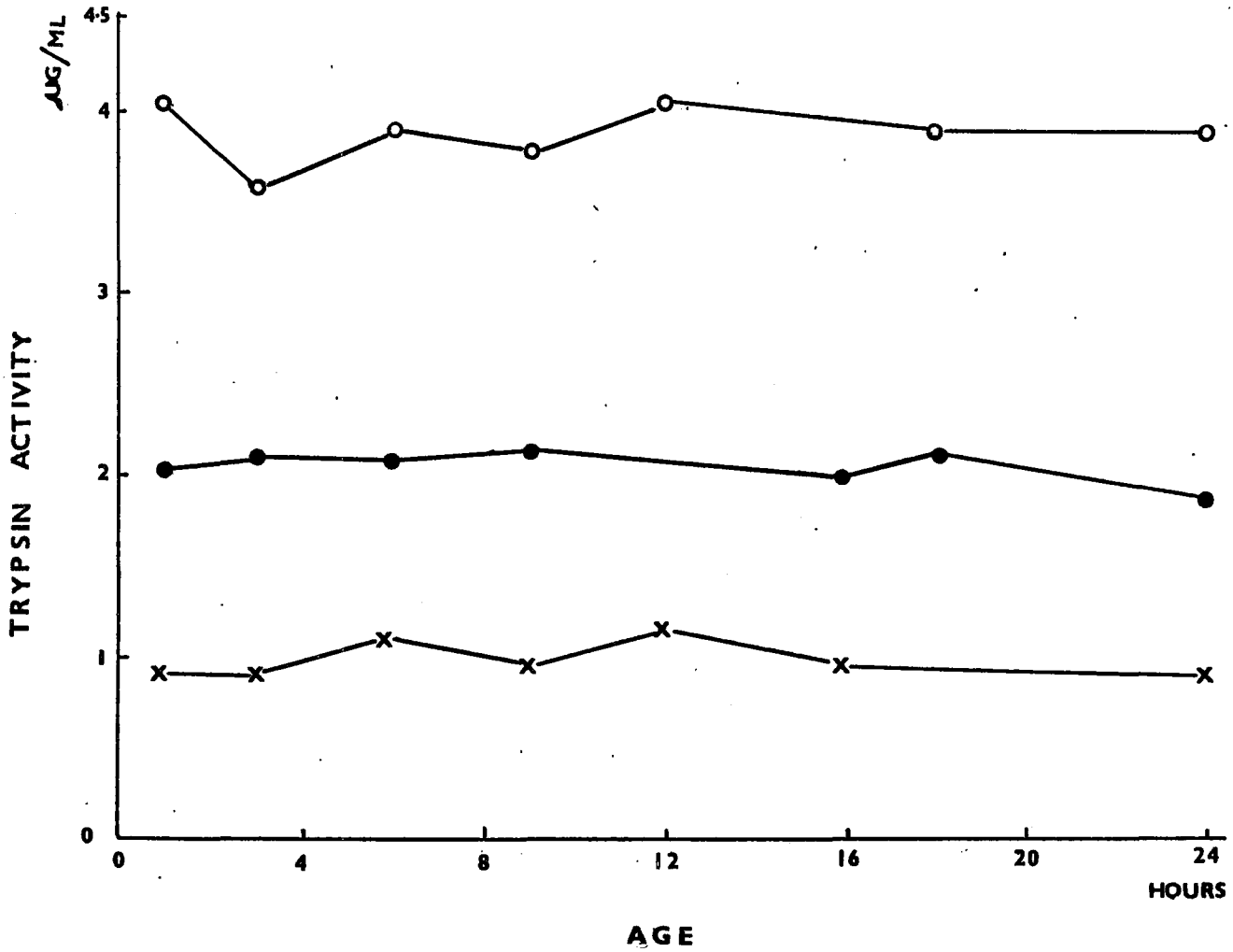
GRAPH 5. The effect of substrate concentration upon trypsin activity of S. venustum and S. rugglesi homogenates at pH 8.0 and 37°C.



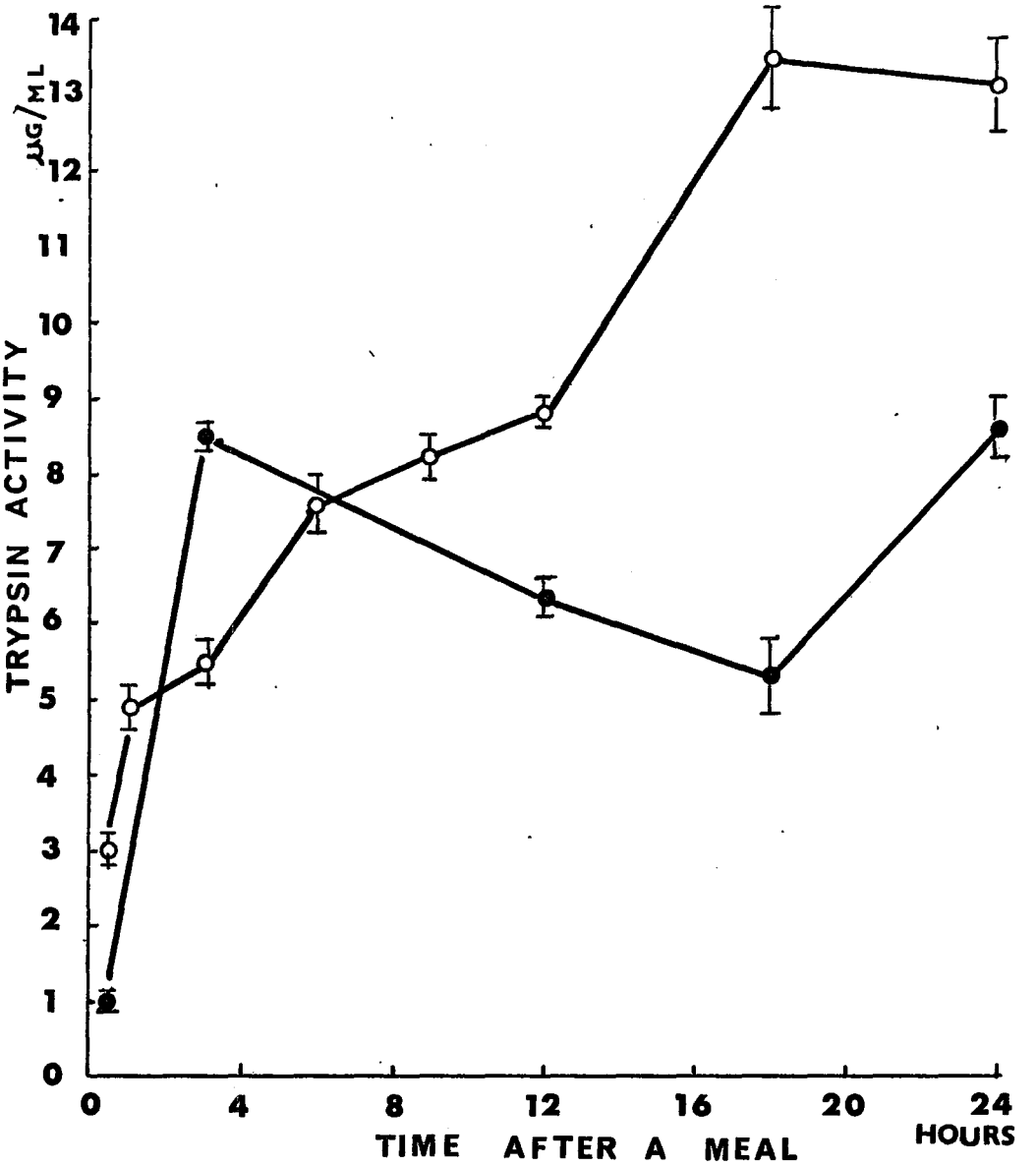
GRAPH 6. Lineweaver-Burk double reciprocal plots of trypsin activty and substrate concentration for two simuliid species.

For S. venustum; Km = 3.1×10^{-3}M

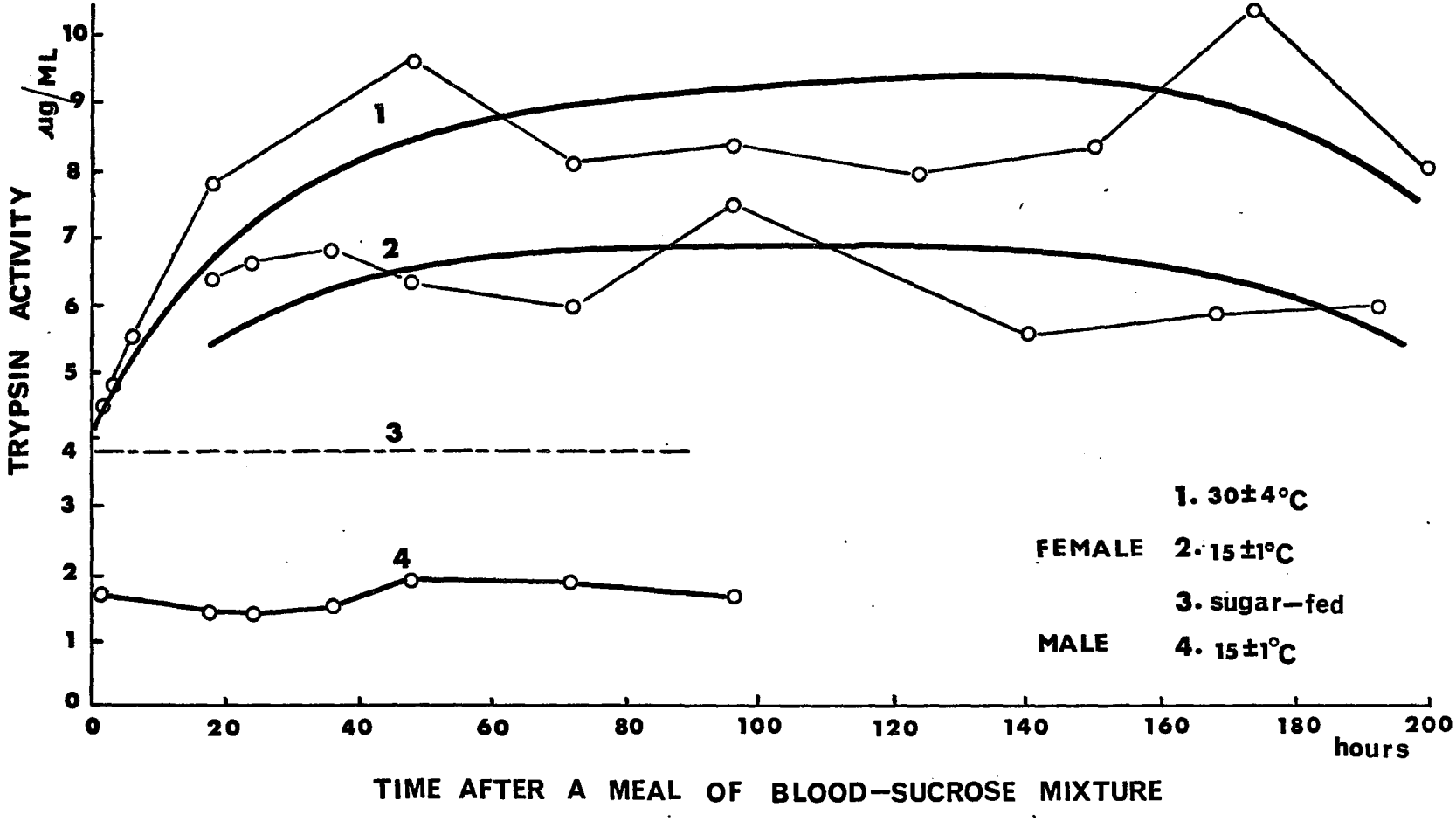
For S. rugglesi; Km = 2.5×10^{-3}M



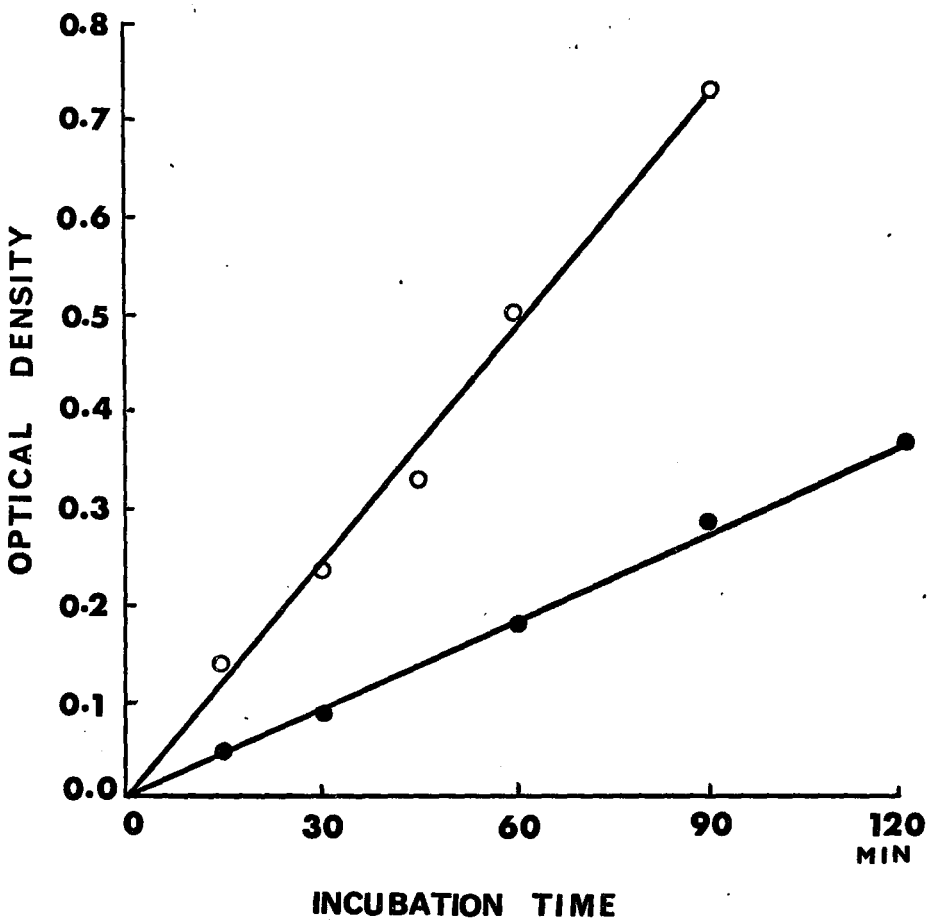
GRAPH 7. Trypsin activity in females of S. venustum, C. dacotensis, P. decemarticulatum at intervals after emergence from the pupae. Flies were provided with dry sugar and water during the experimental period. Symbols: o-o; S. venustum, •-•; C. dacotensis, x-x; P. decemarticulatum.



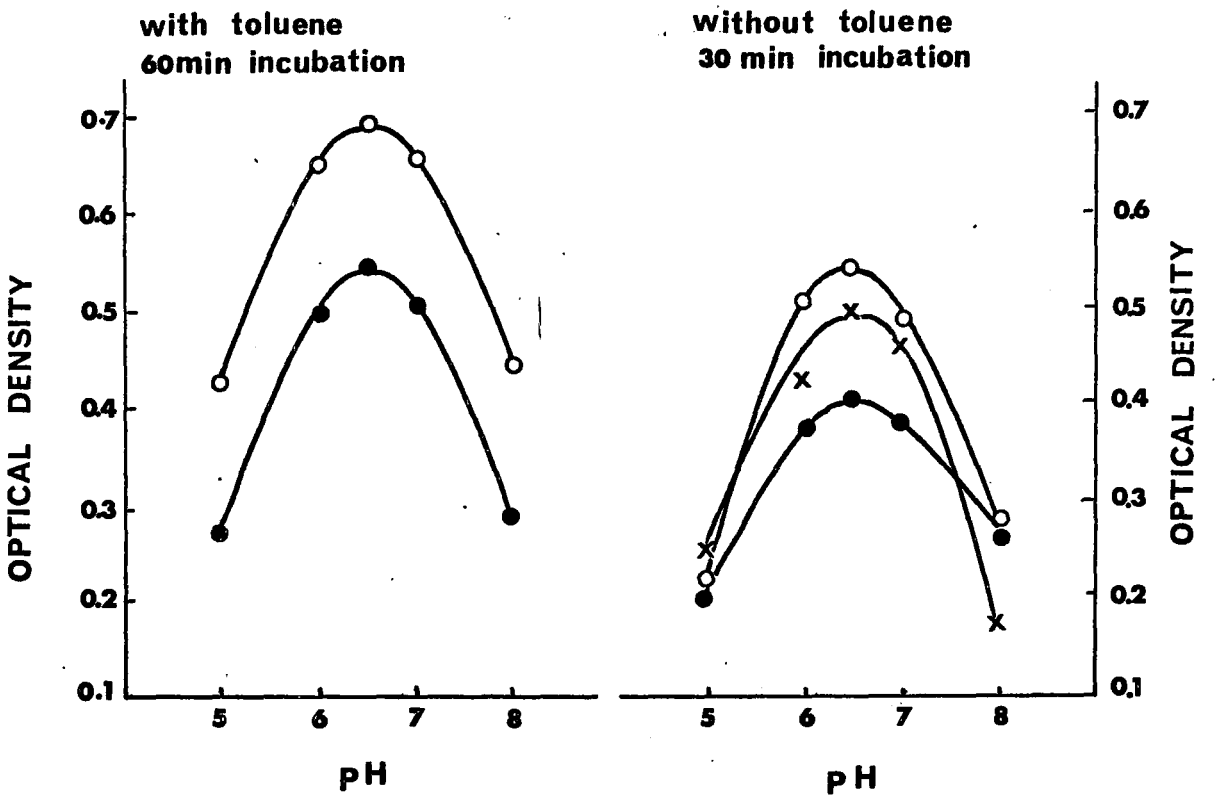
GRAPH 8. Trypsin activity in females of two simuliid species at intervals after blood feeding on birds. Symbols: o-o; S. rugglesi fed on duck, •-•; P. decemarticulatum fed on chicken.



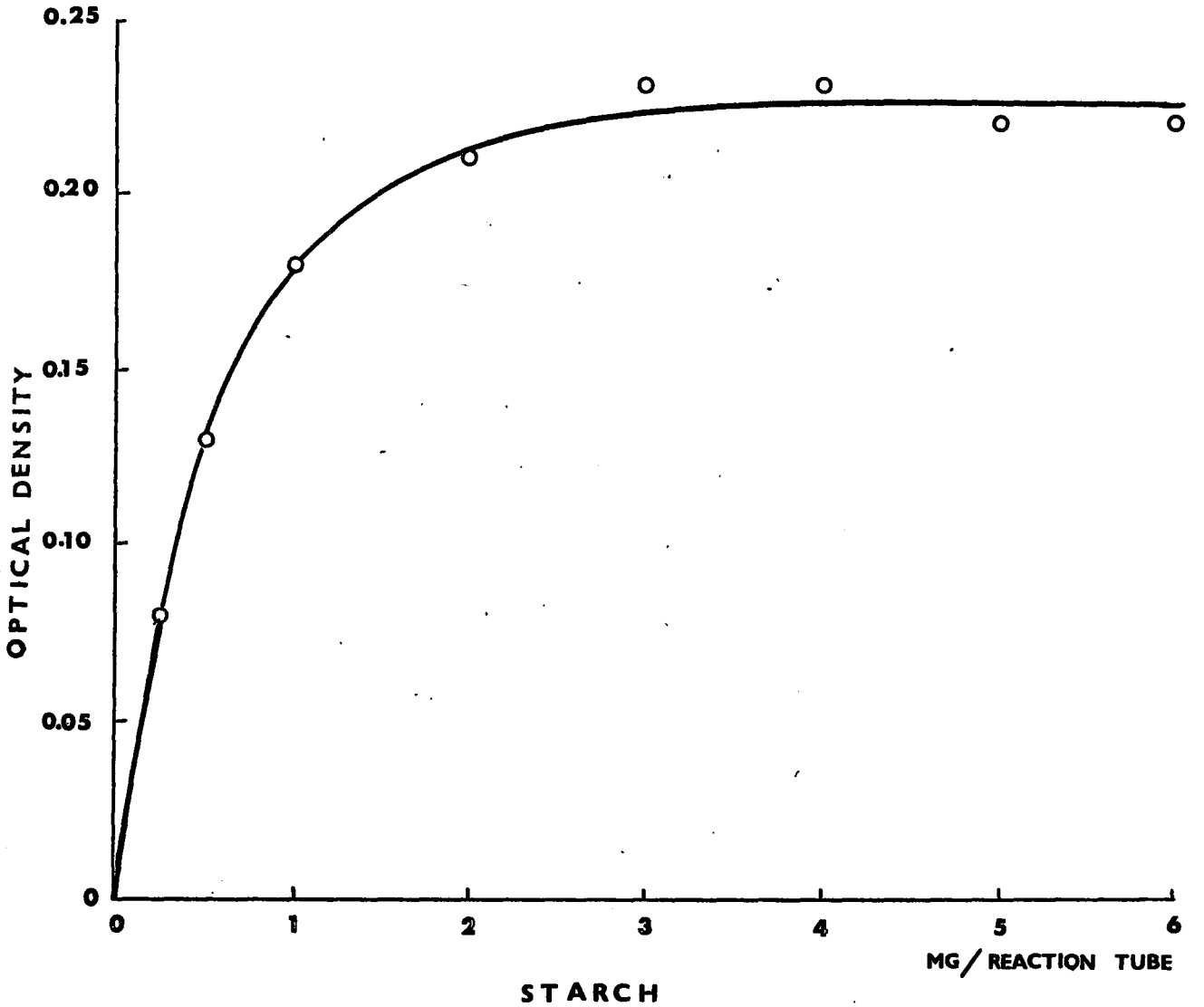
GRAPH 9. Trypsin activity in S. venustum adults at intervals after feeding on a mixture of human blood and sucrose solution. Solid lines in 1 and 2 represent theoretical values obtained by polynomeal-regression.



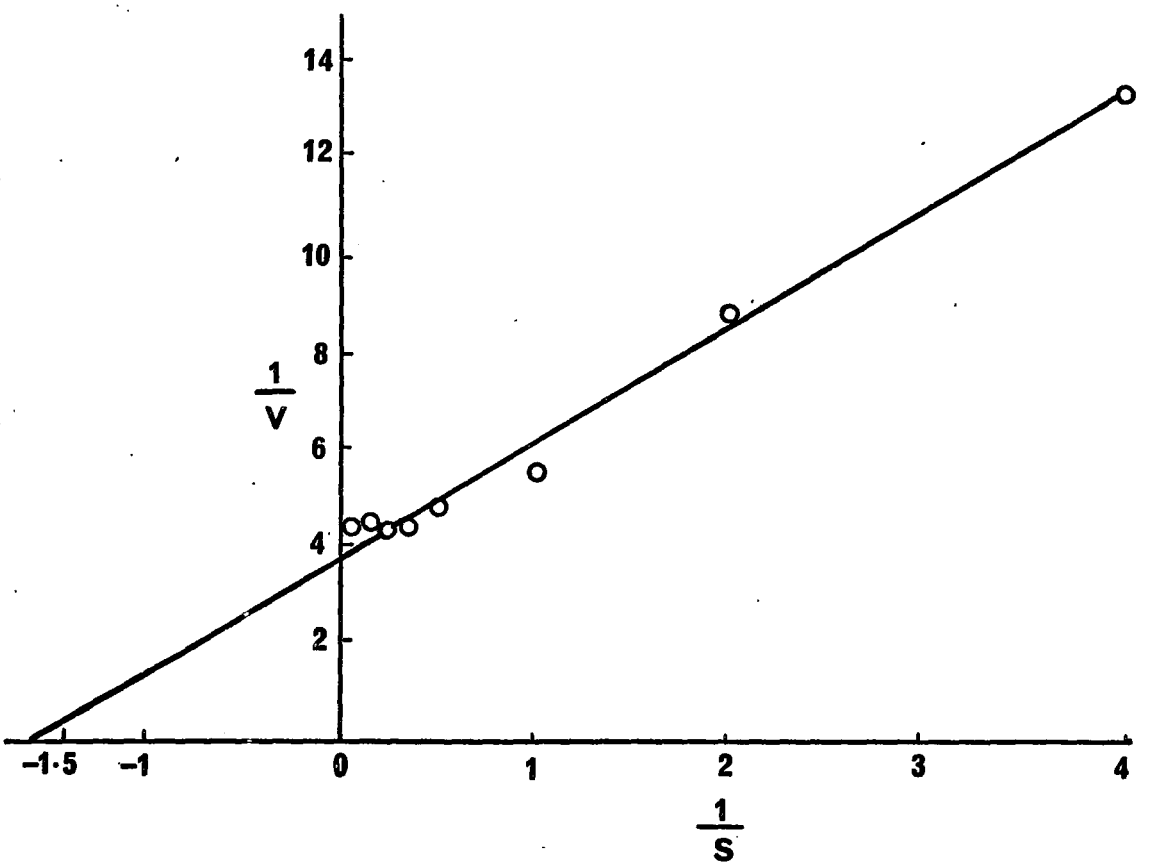
GRAPH 10. Relationship between amylase activity (expressed as optical density) of black-fly homogenates and incubation time at 37°C. Symbols: o-o; S. venustum female, 4 flies/ml, •-•; P. fuscum female, 4 flies/ml.



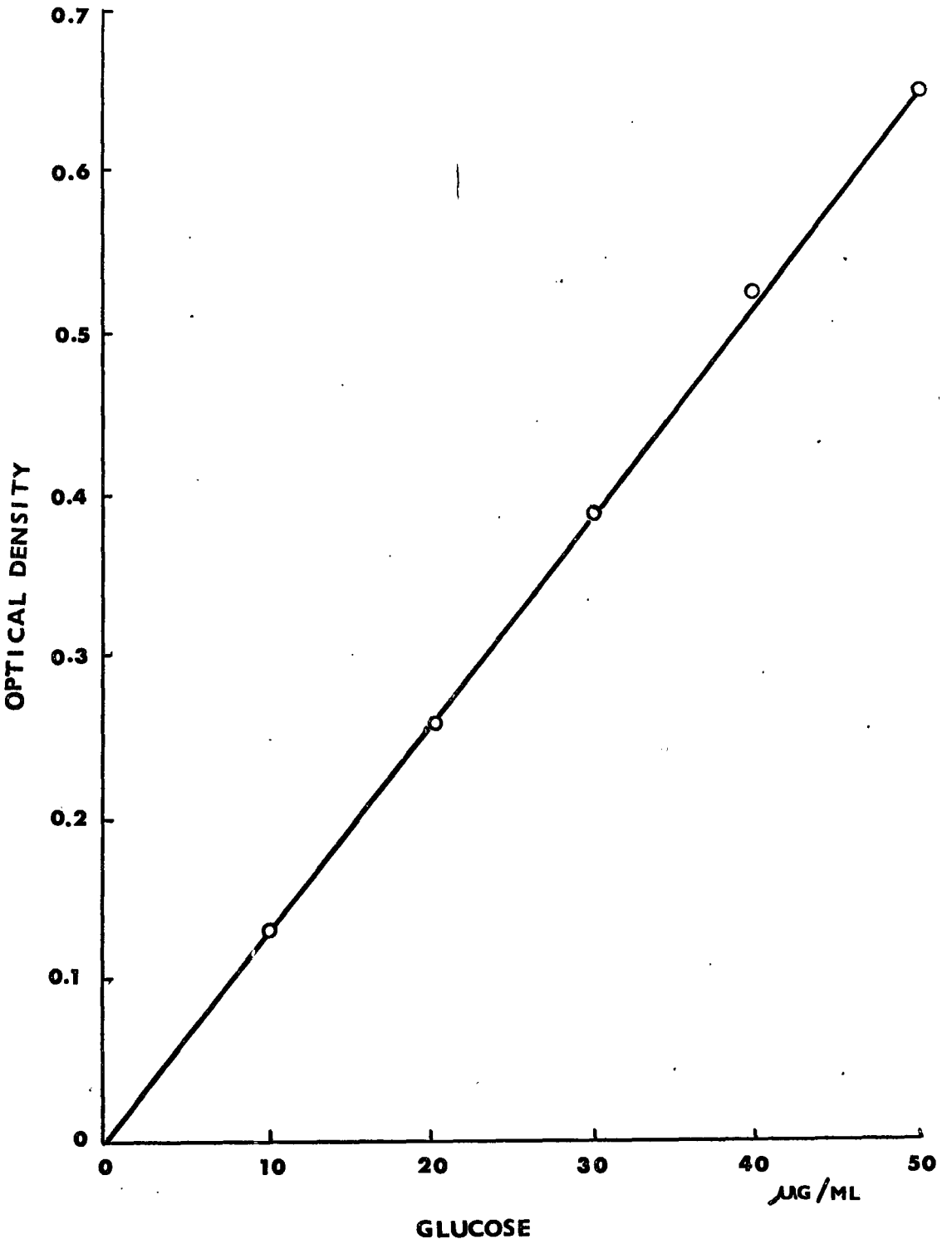
GRAPH 11. The effect of pH upon the amylase activity (expressed as optical density) in homogenates of S. venustum males and females of the same age. Symbols: o-o; Female homogenates, 5 flies/ml, x-x; Female homogenates, 4 flies/ml, •-•; Male homogenates, 7 flies/ml.



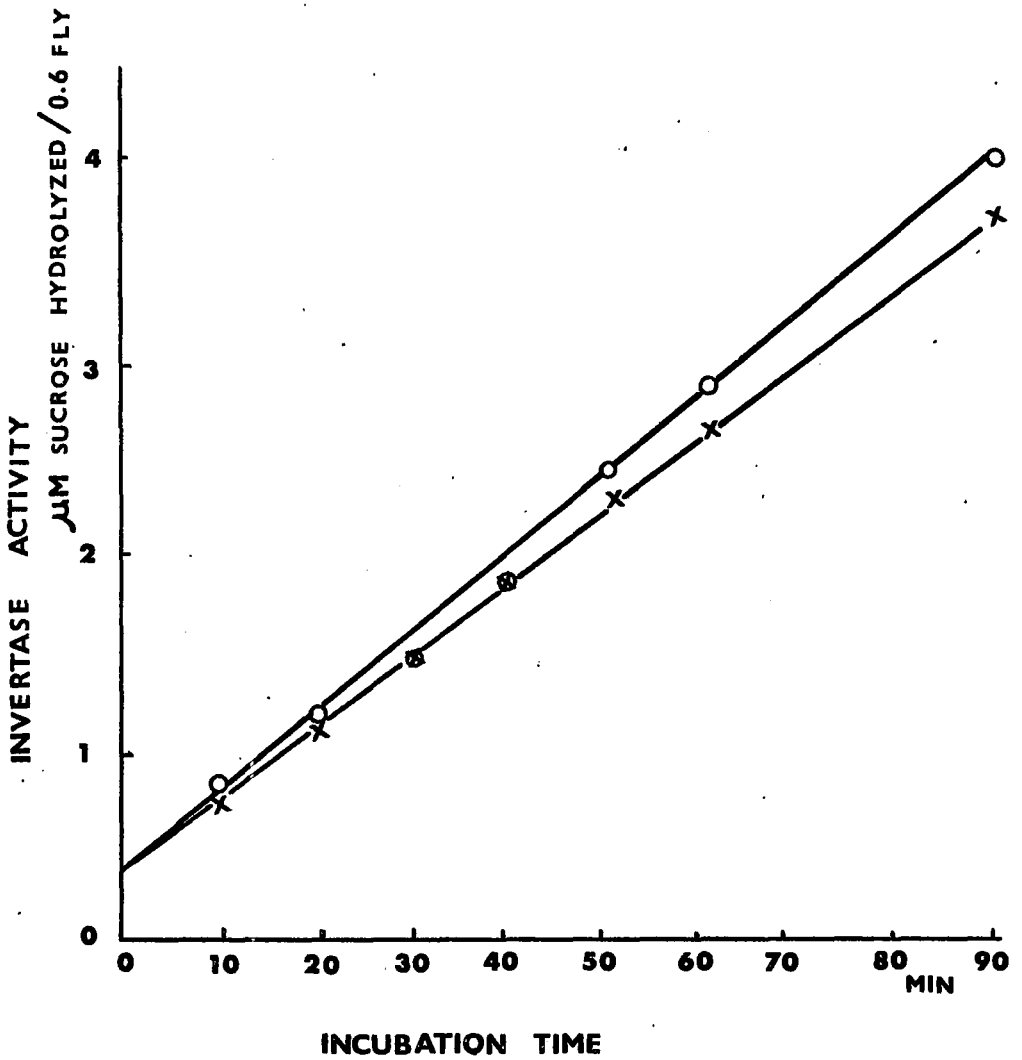
GRAPH 12. The effect of substrate concentration upon the amylase activity (expressed as optical density) in homogenates of S. venustum females. Incubated at 37°C for 30 min.



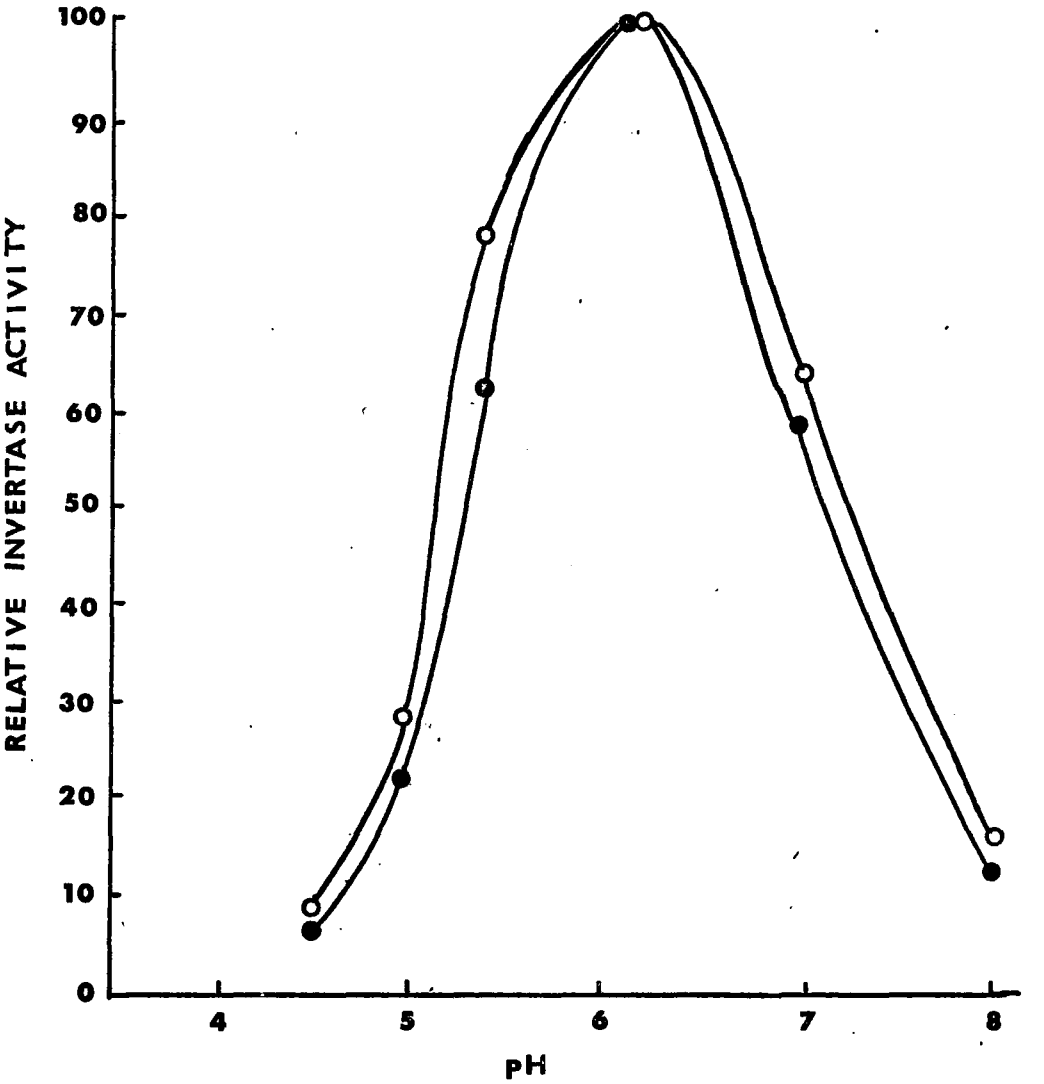
GRAPH 13. Lineweaver-Burk double reciprocal plot of substrate concentration and amylase activity in S. venustum females. Km = 6.5 x 10^{-1} mg/ml.



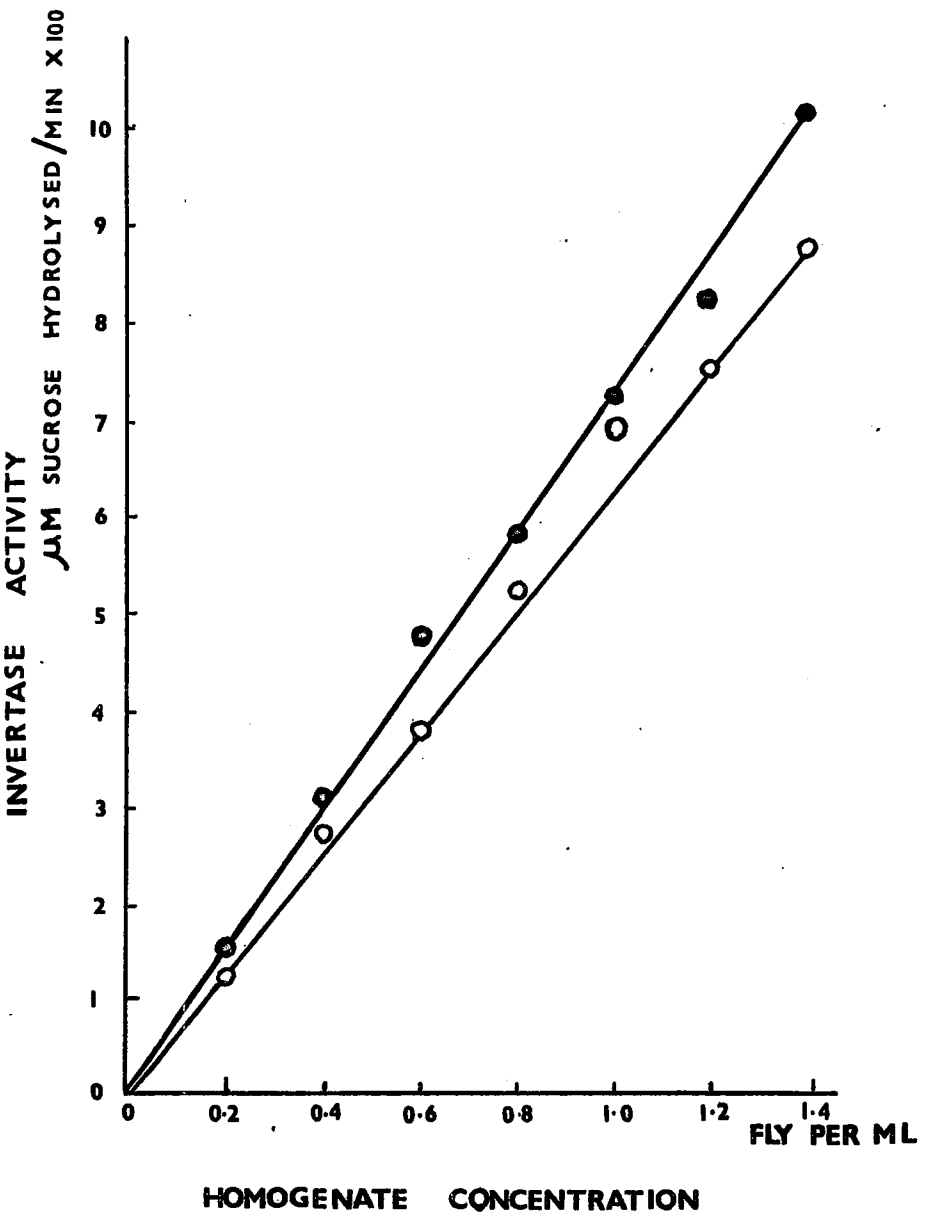
GRAPH 14. Standard curve for glucose concentration, incubated for one hour at 37°C.



GRAPH 15. Relationship between invertase activity in two homogenates of S. venustum females and incubation time at 37°C. Symbols: o-o; No. 1 homogenate, x-x; No. 2 homogenate.



GRAPH 16. The effect of pH upon invertase activity in the homogenates of the midgut and whole body of S. venustum females. Symbols: o-o; Whole fly homogenate, ●-●; midgut homogenate.



GRAPH 17. The relation between invertase activity and homogenate concentration, expressed as the number of flies per ml. Symbols: ●-●; No. 5 homogenate, o-o; No. 7 homogenate.

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