SOURCES OF LARVAL SALIVARY

GLAND SECRETION IN THE

DIPTERAN *CHIRONOMUS TENTANS*

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ABSTRACT

The soluble proteins in the hemolymph, the salivary gland, and the salivary secretion of fourth instar *Chironomus tentans* were examined by disc electrophoresis in acrylamide gels. Of the 11 protein fractions detected in buffered saline extracts of the gland, 10 are present also in the hemolymph. Amino acid isotope incorporation experiments indicate that the protein fractions shared by the salivary gland and the hemolymph are not synthesized in the gland but are synthesized in other larval tissues. Immunochemical studies show that most of these proteins eventually are secreted from the gland. The salivary gland in vivo and in vitro is active in *de novo* protein synthesis. The protein synthesized tends to form large molecular weight aggregates. As demonstrated by radioautography, at least 80% of this protein is secreted from the 30 large cells forming most of the gland. The proteins synthesized in the salivary gland cannot be detected in the hemolymph. The results of this investigation are consistent with a mechanism of secretion formation involving both *de novo* synthesis of some secretion proteins and the selective uptake, transport, and secretion of hemal proteins by the salivary gland.

INTRODUCTION

The primary function of the larval salivary gland in the dipteran *Chironomus* is the elaboration of a viscous secretion. This secretion is used to build a tube in which the larva lives. Tissue-specific puffs, especially the Balbiani rings, on the giant salivary gland chromosomes are believed to reflect gene activities (10, 26) and have been correlated with the secretory process (2, 3, 16–19). It is assumed that such puffs control the synthesis of tissue-specific proteins, i.e. proteins which are synthesized by the gland and by no other larval tissue. Presumably these proteins then are discharged from the gland as secretion. However, experiments from this (17, 18) and other labora-

tories (12, 27, 28) have failed so far to resolve any proteins unique to the salivary secretion of *Chironomus* or related Diptera. To explain the apparent ubiquity of secretion proteins, Laufer and Nakase (18) proposed a transport mechanism for the salivary gland and demonstrated that the gland is capable of sequestering proteins from the hemolymph and secreting them.

Although a part of the secretion arises by transport of hemal proteins, it is also possible that other secretion proteins are synthesized in the salivary gland but, to date, have not been detected in the secretion. In the present paper, we have examined in some detail the tissue distribu-

tion and the origin of the secretion proteins in larval salivary glands of *Chironomus tentans*. The ultimate purpose of these investigations is to correlate, if possible, tissue-specific puffs with specific functions of the larval salivary gland. The results presented here confirm and extend the evidence for the transport mode of secretion formation and also show that other proteins in the secretion are synthesized *de novo* in the salivary gland. These latter proteins are different from the proteins which are transported, and they have characteristics which suggest that they are tissue-specific.

METHODS AND MATERIALS

Experimental Organism

Fourth instar *Chironomus tentans* larvae were used in all experiments. They were reared in the laboratory with continuous light and aeration at a temperature of 21°–23°C. From the time of hatching, the organisms were fed ad libitum a diet of cooked nettle powder, chitin, brewer's yeast, powdered milk, and water (4:1:1:1:4). Development from the egg to the adult requires about 4 wk under these conditions.

Materials

l-Leucine-^{14}C (50 μc/0.0239 mg), *l*-phenylalanine-^{14}C (50 μc/0.0235 mg), and *l*-lysine-^{14}C (237 mc/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Volk Radiochemical Co. (Burbank, Calif.) supplied *d,l*-lysine-^{3}H (300 mc/mmole) and mixed *l*-amino acids-^{14}C (0.8 mc/mg). Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). Freund's complete and incomplete adjuvants were obtained from Difco Laboratories, Detroit, Mich. Silica-gel thin-layer chromatography sheets (Chromagram 6060) and NTB-3 liquid emulsion were purchased from Eastman Kodak Co., Rochester, N.Y. Bio-Gel A 15m was obtained from Bio-Rad Laboratories, Richmond, Calif. Other reagents were also purchased from commercial suppliers.

Preparation of Extracts

Salivary glands were dissected from larvae and were rinsed five times with cold 0.1 M NaCl buffered to a pH of 8.6 with 0.05 M tris (hydroxymethyl) aminomethane (Tris-saline buffer) to remove contaminating hemal proteins. They then were homogenized in all-glass homogenizers in cold Tris-saline buffer. The homogenates were centrifuged at 10,000 *g* for 10 min at 4°C to sediment debris, and the resulting

supernatant fraction was used as the salivary gland extract.

For determination of the apparent molecular size of certain proteins in the salivary gland, extracts were prepared for Bio-Gel A 15m chromatography by homogenizing glands in cold 0.1 M NaCl containing 0.2% sodium desoxycholate. The homogenate was centrifuged for 1 hr at 105,000 *g* at 4°C. The resulting supernatant fluid was applied to a Bio-Gel A 15m column, 60 × 2.5 cm, equilibrated with 0.1 M NaCl containing 0.2% sodium desoxycholate. 1.5-ml fractions were collected at a flow rate of 30 ml/hr. Total hexose in the fractions was determined by the anthrone colorimetric assay (32). Absorption was measured at a wavelength of 620 mμ.

Secretion was obtained by a modified (18) method of Defretin (7). Larvae were allowed to secrete onto reagent-grade sand. The sand was rinsed five times with distilled water and was extracted with cold Tris-saline buffer. The extract was centrifuged at 4°C for 10 min at 10,000 *g* before use.

Hemolymph was collected into capillaries from larvae by cutting the posterior proleg and was centrifuged at 4°C for 10 min at 10,000 *g* before use.

Protein was determined by the method of Lowry et al. (20) with bovine serum albumin as standard.

Disc Electrophoresis

Proteins in extracts of the salivary gland and the hemolymph were examined and compared by the extremely sensitive disc electrophoresis technique (6, 24). Usually 200–400 μg of salivary gland extract or hemolymph protein were applied to the standard 7.5% acrylamide column. The microsomes in the 10,000 *g* Tris-saline extract of salivary glands tend to plug the pores in the acrylamide gel, thereby prolonging the time required to achieve electrophoretic separation. Therefore, before electrophoresis, this extract was freed of microsomes by centrifugation at 105,000 *g* for 1 hr at 4°C. The removal of microsomes has no observable effect on the pattern of proteins resolved from the gland extract. Electrophoretic separations were obtained at 4°C with a constant current of 5 ma/column. Electrophoresis was terminated when the tracking dye, bromphenol blue, had migrated 4 cm into the separating gel. Proteins were fixed and stained with 1% Amido Schwartz in 7.5% acetic acid. After the proteins had been stained, the excess dye was removed by placing the gels overnight in 7.5% acetic acid. Hemoglobins were visualized with benzidine (5). R_f denotes the ratio of the distance traveled by a protein band to that traveled by the tracking dye.

When radioactively labeled proteins were separated by disc electrophoresis, the gels first were stained in the usual manner with Amido Schwartz, and the R_f values of the separated proteins were determined.

The gels then were frozen on dry ice and were cut sequentially into 2-mm slices. Acrylamide in the gel slices was dissolved by the H_2O_2 method of Moss and Ingram (23), and the residue was quantitatively transferred to Whatman 542 filter paper for liquid scintillation counting.

Preparation of Antisera and Immunochemistry

Antigens in extracts of the hemolymph, the salivary gland, and the secretion were examined and compared by quantitative precipitin reactions, by Ouchterlony gel double diffusion analysis, and by immunoelectrophoresis (11).

Antisera were prepared by administering hemolymph or salivary gland extract to rabbits in five subcutaneous injections. Equal volumes of the respective extract and Freund's complete adjuvant were used for the sensitizing injection. Booster injections consisting of equal volumes of extract and Freund's incomplete adjuvant were given at weekly intervals beginning 2 wk after the sensitizing injection. A total of 150 mg of hemal protein or 30 mg of salivary gland extract protein was administered to each rabbit.

Antiserum to secretion was prepared by injecting a Tris-saline extract of the secretion intravenously into rabbits. Injections were given daily for 5 consecutive days. 1 wk later, daily injections were given again for 5 consecutive days. A total of 20 mg of protein was administered to each rabbit in three such series of injections.

In all cases the rabbits were bled 8 days after the final injection. Sera were harvested after centrifugation and were stored at −20°C until used.

Disc electrophoresis in combination with an agar trench technique was used as a method for immunoelectrophoresis. After electrophoresis, the unfixed and unstained acrylamide gel was immersed in 0.8% agar in 0.05 M Tris buffer, pH 7.4. Antisera were placed in 4 × 15 mm trenches cut parallel to and 1 cm from the gel. The Petri plates then were incubated at 4°C for 7 days.

In Vivo Incorporation of Isotopes

Usually 0.1 µc of amino acids-^{14}C in a volume of 2.5 µl was injected into each larva by puncturing one of the posterior prolegs with a microcapillary glass needle attached to a 50 µl syringe. The wound was closed with a six-gauge surgical silk ligature. At the time intervals specified in the legends to the tables and figures, the salivary glands and the hemolymph were collected from the larvae and were prepared for the determination of radioactivity present in protein. In some experiments, the larvae were ligatured behind the fourth body segment with surgical silk

before injection of the amino acid isotope. This procedure effectively isolates the salivary glands from the portion of the hemocoel behind the ligature which contains the labeled amino acids.

Organ Culture and In Vitro Incorporation of Isotopes

Larvae were surface sterilized by rinsing for 30 sec in 70% ethanol containing 0.05% $HgCl_2$. The salivary glands were dissected aseptically into Schneider's defined culture medium (30), rinsed three times with the medium, and incubated in shallow depression slides at room temperature. Four to 10 glands were incubated per 0.5 ml of the medium.

As a precursor for protein, 4 µc of an amino acid-^{14}C were added to each ml of the culture medium. The homologous, unlabeled amino acid was omitted from the medium. Puromycin, when used to inhibit protein synthesis, was added to the organ culture to give a final concentration of 100 µg/ml in the medium and was administered 10 min before the addition of isotope. The glands were exposed to the labeled amino acid for the intervals denoted in the legends to the tables and figures, and then they were prepared for the determination of radioactivity present in protein.

Radioautography was used to study the dynamics of protein synthesis and secretion in vitro. Salivary glands were given a 15-min pulse of lysine-^{3}H, 10 µc/ml culture medium. Incorporation of the label was terminated by the addition of a 100-fold excess of unlabeled lysine to the culture. At intervals thereafter, the glands were removed from culture and were fixed overnight at 4°C in 1% formaldehyde buffered to a pH of 7.4 with 0.1 M phosphate. The fixed glands were rinsed in 0.1 M phosphate, pH 7.4, dehydrated in ethanol, cleared in benzene, and embedded in paraffin. Serial sections were cut at 5 µ. The sections were hydrated through a graded series of ethanol into water and were rinsed for 20 min in two changes of cold 5% trichloroacetic acid containing unlabeled lysine to remove acid-soluble materials. In some cases, they were rinsed for an additional 10 min in 5% trichloroacetic acid at 90°C to remove nucleic acids. The sections then were coated with Kodak NTB-3 liquid emulsion according to the technique of Kopriwa and Leblond (15). Emulsion exposure time was 8 days. Silver grains were counted at a magnification of 1250 with an ocular reticle in which each subdivision enclosed an area of 23 μ^2.

The extent of lysine conversion to other metabolites after exposure of salivary glands in vitro to lysine-^{14}C was examined. The glands were removed from organ culture and were homogenized in 10% trichloroacetic acid containing unlabeled lysine. The recovery of radioactivity as lysine was determined by

hydrolyzing the washed precipitates in 6 N HCl for 10 hr in sealed glass tubes at 110°C. HCl was removed by vacuum desiccation over NaOH. The residue was dissolved in water containing 1.0 mg/ml authentic lysine and was subjected to thin-layer chromatography on silica gel. The chromatograms were developed in two dimensions with n-butanol-acetic acid-water (80:20:20) as primary solvent and n-propanol-water (70:30) as secondary solvent. The separated amino acids were visualized with ninhydrin, and the radioactivity in the lysine spot was determined.

Counting Procedure

Hemolymph protein samples were prepared for counting by the filter paper technique of Mans and Novelli (21). In some experiments, the hemolymph was dialysed for 24 hr at 4°C against 0.1 M NaCl to remove unincorporated labeled amino acids. The salivary gland protein samples were treated, as described by Siekevitz (31), with unlabeled amino acids in the trichloroacetic acid washes. The washed precipitates were dissolved in 1.0 N NaOH, and a suitable aliquot was transferred to Whatman 542 paper for counting. The filter papers were counted in 10 ml of scintillator (4 g of 2,5-diphenyloxazole and 0.5 g of 2-p-phenylenebis 5-phenyloxazole per liter of toluene). Aliquots of fractions from the Bio-Gel A 15m column as well as acid-soluble material in the trichloroacetic acid washes were counted in Bray's liquid scintillator (4). All counting was done in a Nuclear-Chicago Liquid scintillation spectrometer (Nuclear-Chicago Corporation, Des Plaines, Ill.). Efficiencies were determined by internal standardization. When the activity is expressed as disintegrations per minute (dpm), the efficiencies are corrected to 100%.

RESULTS AND CONCLUSIONS

Protein Composition of the Salivary Gland, the Hemolymph, and the Secretion

11 major protein fractions are resolved when extracts of salivary glands are subjected to disc electrophoresis (Fig. 1). 10 of these fractions also appear to be present in the hemolymph. That is, 10 of the 11 protein bands detected in extracts of the salivary gland have identical R_f values as protein fractions in the hemolymph (Table I). The fact that the resolved protein bands are additive when mixtures of hemolymph and salivary gland extract are subjected to disc electrophorese also suggests that some proteins are shared by this gland and the hemolymph.

Because of their red color and their staining properties with benzidine, nine of the protein fractions in the hemolymph tentatively are identified as hemoglobins. Four of these hemoglobins are present in the salivary gland (Table I). Moreover, the major hemoglobin in the gland, R_f 0.550, is a minor fraction in the hemolymph (Fig. 2). Since equal amounts of hemolymph and salivary gland protein were applied to the acrylamide columns shown in Fig. 2, on a per protein basis the hemoglobin with R_f 0.550 appears to be more concentrated in the gland than in the hemolymph. It is important to note that some hemal proteins, including five of the hemoglobins, are not present in detectable quantities in the salivary gland (Table I).

That proteins are shared by the salivary gland and the hemolymph was confirmed further by immunochemical techniques. An antiserum to salivary gland extract shows a complex pattern of many immune precipitate lines when reacted in Ouchterlony double diffusion with the homologous gland extract (Fig. 3). Only two of these immune precipitate lines survive when this antiserum is exhaustively absorbed both with hemolymph and
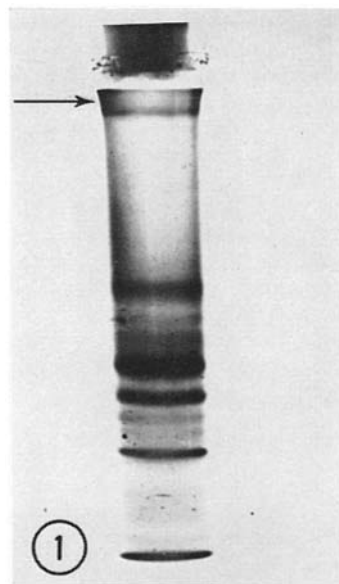

FIGURE 1 Disc electrophoresis of a Tris-saline extract of salivary glands. 250 µg of protein in the extract were applied to the acrylamide column. Anode is toward the bottom of the figure. The arrow denotes the approximate location of the protein band which is not present in the hemolymph (R_f 0.042).

TABLE I

Disc Electrophoresis of a Tris-Saline Extract of Salivary Glands and of Hemolymph; R_f Values of the Separated Protein Fractions

Material: Gland extract Stain: Benzidine	Gland extract Amido Schwartz	Hemolymph and gland extract Amido Schwartz	Hemolymph Benzidine	Hemolymph Amido Schwartz
	0.042	0.042		
	0.057	0.055		0.051
		0.075		0.077
		0.100		0.103
	0.169	0.162		0.154
		0.405	0.405	0.410
	0.430	0.432		0.440
		0.480		0.485
0.550	0.545	0.545	0.530	0.540
	0.575	0.565		0.565
	0.610	0.615		0.615
0.667	0.665	0.670	0.660	0.680
	0.730	0.730	0.735	0.745
		0.790	0.785	0.795
0.820	0.830	0.825	0.830	0.830
0.880	0.890	0.885	0.875	0.885
		0.930	0.930	0.935
		0.990	0.990	0.990

Preparation of extracts, conditions for electrophoresis, and method for calculating R_f values are given in the text under Methods and Materials. 300 μg of either gland or hemal protein were added to the acrylamide column. 100 μg of hemal protein were added to 250 μg of gland protein to form the hemolymph-gland mixture. Amido Schwartz was used to stain proteins; hemoglobins were stained with benzidine.

with extracts of larvae from which the salivary glands previously had been removed (Fig. 4). Thus, the gland is antigenically heterogeneous, but most of the antigens also are present in the hemolymph and in other larval tissues. Presumably only the antigens forming the nonabsorbable immune precipitates are unique to the gland.

Immunoelectrophoresis was used to correlate the protein fractions resolved by disc electrophoresis with the antigens in the salivary gland. The results are shown in Fig. 5. Although it is not possible with this technique to assign a specific antigen to a specific protein fraction, the immunoelectrophoretic results are consistent with the data presented in Table I. Thus, as shown in Fig. 5, the determinants for the gland-specific antigens do not migrate very far, if at all, into the separating gel during disc electrophoresis of the salivary gland extract; as shown in Table I, the one protein fraction present in the gland but absent in the hemolymph (R_f 0.042) also does not migrate very far into the separating gel. As

also shown by comparing Fig. 5 with Table I, the major antigens shared by the salivary gland and by other larval tissues are present in the same regions of the gel after electrophoresis as the protein fractions shared by the gland and the hemolymph.

Immunoelectrophoresis in our hands is not so sensitive as Ouchterlony immunodiffusion, probably because of the relatively small amount of protein that can be applied to the acrylamide column. Therefore, only the major antigens in the gland extract are resolved. Ouchterlony double diffusion analysis shows that two precipitation lines develop when the gland extract is reacted with the absorbed antiserum to gland (Fig. 4). Only one of these lines is detected by immunoelectrophoresis. The other immune precipitate can be resolved by immunoelectrophoresis as a faint arc also near the spacer gel if the acrylamide column is heavily loaded with gland extract protein before electrophoresis.
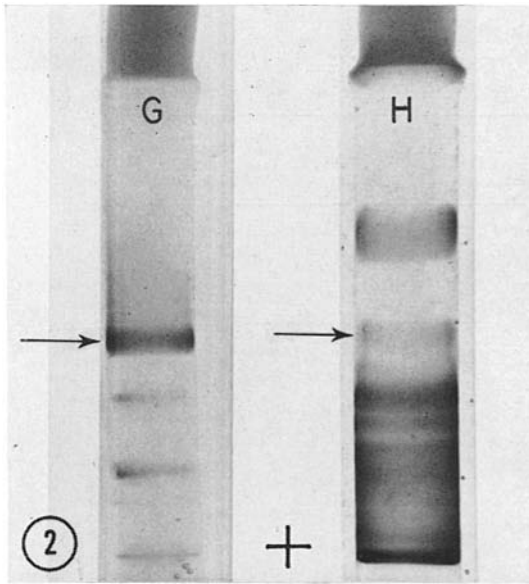
Because of certain peculiar characteristics

Figure 2 Disc electrophoresis of hemolymph and a Tris-saline extract of salivary glands; hemoglobins stained with benzidine. Hemolymph was diluted in Tris-saline buffer, and 250 μg of hemal or gland extract protein were applied to each column. Anode is denoted by plus sign. The major benzidine-staining band in the gland and the corresponding band in the hemolymph are indicated by arrows. *H*, hemolymph; *G*, gland extract.

which will be discussed later, the salivary secretion is extremely difficult to examine by disc electrophoresis. Therefore, an immunochemical technique was employed to determine whether the protein antigens shared by the salivary gland and the hemolymph are present in the secretion and, if they are, whether these proteins can be synthesized in the complete absence of the salivary gland. Antisera were prepared to extracts of the salivary glands, to extracts of the secretion, and to the hemolymph and were reacted immunochemically with the hemolymph. Each of the antisera form immune precipitates with hemolymph with titers of 1:8 for antigland, 1:32 for antisecretion, and 1:64 for antihemolymph (Fig. 6). Furthermore, amino acid-^{14}C incorporation experiments show that hemal proteins which were synthesized in vivo in the complete absence of the salivary gland also form immune precipitates with each of the antisera (Table II). The radioactivities in the immune precipitates are proportional to the titers of the respective antisera. These results strongly suggest that proteins in the hemolymph, which can be synthesized in the complete absence of the salivary gland, are present both in the salivary gland and in the secretion. Indeed, all of the antigens detectable in the secretion are present also in the hemolymph (18). That is,
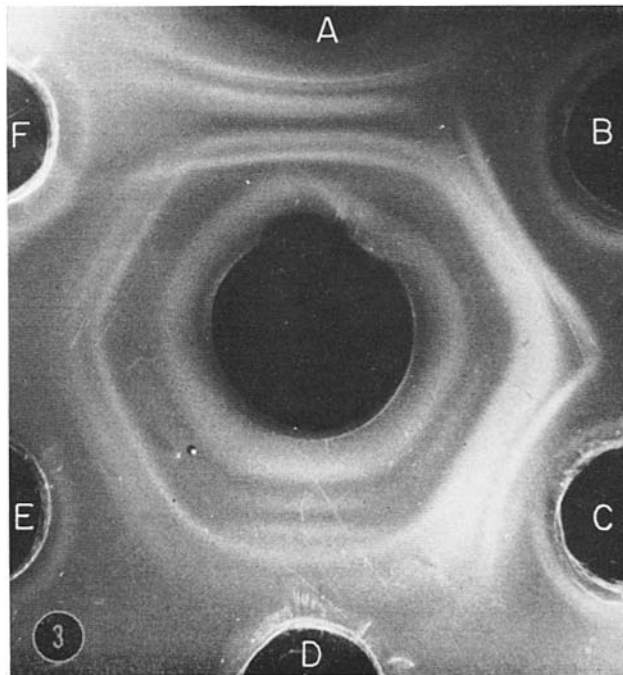

Figure 3 Ouchterlony immunodiffusion plate demonstrating the immunological specificity of antiserum prepared to a Tris-saline extract of salivary glands. The peripheral reservoirs contained the following: *A*, Tris-saline extract of salivary glands; *B*, Tris-saline extract of whole larvae; *C*, same extract as *B*, 1:2 dilution; *D*, Tris-saline extract of larvae from which the salivary glands previously had been removed; *E*, same extract as *D*, 1:2 dilution; *F*, same extract as *D*, 1:4 dilution. The central reservoir contained antiserum to gland extract.
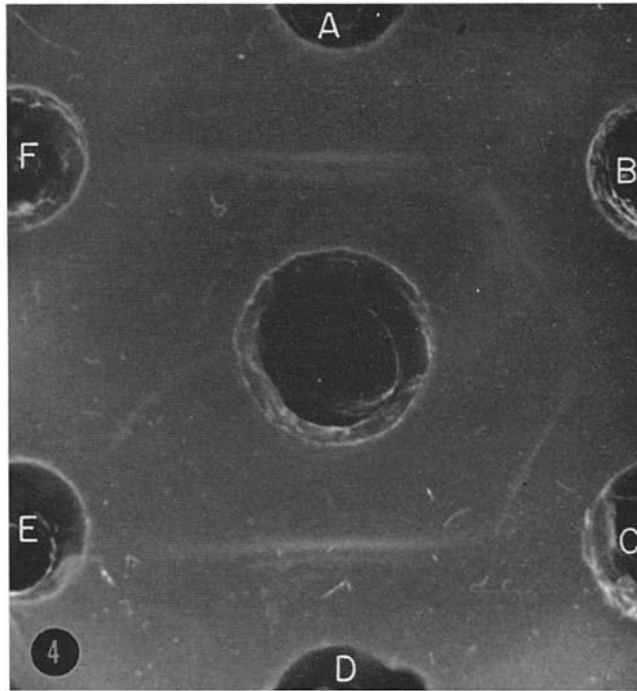
FIGURE 4 Ouchterlony immunodiffusion plate demonstrating the immunological specificity of antiserum prepared to a Tris-saline extract of salivary glands after absorption. The peripheral reservoirs contained the following: A, Tris-saline extract of salivary glands; B, Tris-saline extract of whole larvae; C, same extract as B, 1:2 dilution; D, same extract as A; E, Tris-saline extract of larvae from which the salivary glands had been removed; F, same extract as E, 1:2 dilution. The central reservoir contained antiserum to gland extract which had been exhaustively absorbed both with hemolymph and with extracts of larvae from which the salivary glands had been removed. 2 ml of the antiserum were absorbed in three consecutive treatments, each with 25 mg of hemolymph, and then in two treatments with Tris-saline extracts from 25 larvae containing no salivary glands. Absorptions were performed as recommended by Kabat and Mayer (11) and were continued until all cross-reacting material was precipitated from the antiserum.

although there are antigens in the gland which are not present in the hemolymph or in other larval tissues (Figs. 4 and 5), these antigens can not be detected in the secretion.

De Novo Synthesis of Secretion Proteins

One or more of the following mechanisms could account for all of the preceding observations concerning the formation of the salivary secretion. (a) The secretion is derived entirely by the selective uptake and subsequent transport across the gland of hemal proteins. (b) Secretion proteins are synthesized by the gland, but the same proteins also are synthesized by other larval tissues and, therefore, are present in the hemolymph. The possibility that these proteins are synthesized in the salivary gland and are transferred to both

the gland lumen and the hemocoel must also be considered. (c) Some secretion proteins are derived by the selective uptake and transport of hemal proteins. Other secretion proteins are synthesized *de novo* only in the salivary gland. They cannot be detected in the secretion by immunochemical techniques because they are secreted in quantities below the limits of immunochemical detection, or because they lose their antigenic properties when secreted from the gland.

An in vitro organ culture system was established to examine protein synthesis in the salivary gland and to distinguish among the above possibilities. With this system any protein uptake and transport activity can be separated from the protein synthetic activity of the gland.

A preliminary analysis (8) of the ribonucleic

acid synthetic capacity and the cellular fine structure revealed no important differences between control salivary glands and glands that had been cultured in vitro for the time intervals used in the following experiments. Incorporation of leucine-^{14}C, which is representative of the other amino acids tested, is linear with respect to time for at least 3 hr (Fig. 7), indicating that the salivary gland in vitro actively incorporates amino acids into material which is insoluble in both hot and cold trichloroacetic acid and in organic solvents. Hot trichloroacetic acid removes at most 1% of the incorporated radioactivity. Thus, most of the acid-insoluble radioactivity is
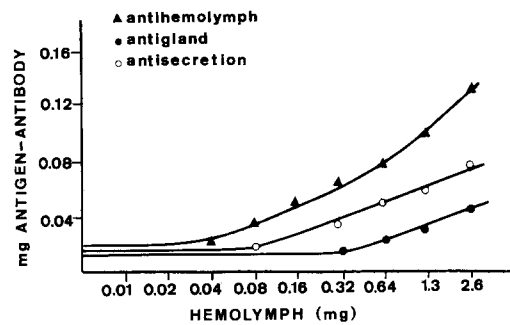

FIGURE 6 Quantitative immunological analysis of antisera to gland extract, to hemolymph, and to secretion. Serial two-fold dilutions of hemolymph were made in 0.1 M NaCl. Undiluted hemolymph in this experiment contained 5.12 mg protein/0.1 ml. To 0.1 ml of each antiserum was added 0.1 ml of hemolymph containing the protein concentration indicated. After completion of precipitation, the protein in the washed immune precipitates was determined by the colorimetric procedure of Lowry et al. (20). The titers to hemolymph of the antisera are 1:8 for antigland (0.64 mg hemal protein), 1:32 for antisecretion (0.16 mg hemal protein), and 1:64 for antihemolymph (0.08 mg hemal protein).

FIGURE 5 Immunoelectrophoresis of salivary gland extract. 400 µg of protein in a Tris-saline extract of salivary glands were subjected to disc electrophoresis. Anode is toward the bottom of the figure. At the conclusion of the run, the acrylamide column was sliced longitudinally; one-half was fixed and stained with Amido Schwartz, and the other half was used for immunodiffusion. The right trench contained antiserum to gland extract; the left trench contained the same serum, but exhaustively absorbed as described in the legend to Fig. 4. The arrow denotes the one precipitation line, near the spacer gel, surviving the absorption. Composite photograph with the fixed and stained half gel substituted for the unfixed half.

not in the form of amino-acyl transfer ribonucleic acid. When glands in vitro are exposed to lysine-^{14}C, more than 80% of the incorporated label cochromatographs with authentic lysine following acid hydrolysis of the trichloroacetic acid-insoluble material (Table III). These results and the fact that incorporation, but not uptake, of labeled lysine is almost completely inhibited by puromycin (Table IV) strongly suggest that the material synthesized is protein.

The proteins synthesized by the salivary gland in vitro separate as two radioactive peaks during disc electrophoresis (Fig. 8). One peak occurs within the spacer gel, and the second peak is just within the separating gel (R_f 0.0–0.06). If the acrylamide column is sliced carefully, it can be shown that the radioactivity in the peak corresponding to R_f 0.0–0.06 is confined to the protein fraction with R_f 0.042. As shown in Table I, this is the only protein fraction in the salivary gland which is not present also in the hemolymph, and it appears to be synthesized in the gland. In Fig. 8, the glands were exposed to the labeled amino acids for 4 hr. With exposure times as short as 15 min, the labeled proteins separate in the same two radioactive peaks. We have never observed any significant synthesis in vitro of the protein fractions in the R_f range 0.05–1.0.

TABLE II

Immunological Precipitation of Hemal Proteins from Ligatured Larvae Injected with Mixed Amino Acids-^{14}C

	Antiserum		
Antigen	Antihemolymph	Antisecretion	Antigland
	total dpm in precipitate		
Test hemolymph (0.31 mg protein)	511	380	71
Test hemolymph (0.15 mg protein)	346	164	43
Control hemolymph with labeled amino acids (0.61 mg protein)	10	10	10

Larvae were ligatured behind the fourth body segment with surgical silk. This procedure isolates the posterior portion of the hemocoel from the salivary glands. 0.1 μc of the labeled amino acids was injected into the hemocoel of each larva. 15 hr later, the hemolymph was collected, pooled, and dialyzed against 0.1 M NaCl. To 0.05 ml of each antiserum was added 0.05 ml of hemolymph containing the protein indicated. After the completion of precipitation, the radioactivity in the washed precipitates was determined. Undiluted hemolymph collected from the hemocoel behind the ligature contained 230 dpm/μl and 12.5 μg protein/μl. Essentially no radioactivity was precipitated when this hemolymph was reacted with control preimmune sera. As a control for trapping of free amino acids-^{14}C in the immune precipitate, unlabeled hemolymph from uninjected larvae was brought to the same specific activity as the test hemolymph with amino acids-^{14}C and then was reacted with the antisera. To show that there was negligible trapping of nonspecific protein in the antigen-antibody complex, unlabeled hemolymph from uninjected larvae was brought to at least the same specific activity as the test hemolymph with human serum albumin-^{125}I before reaction with the antiserum. There was no ^{125}I activity in the immune precipitates. Hemolymph collected from the portion of the larva anterior to the ligature had less than 1% of the ^{14}C activity of the hemolymph from the posterior portion, showing that the hemocoel behind the ligature was effectively isolated from the salivary glands.

The distribution after disc electrophoresis of the labeled proteins in the salivary gland extract is not an artifact of the organ culture system. An identical pattern is obtained if the labeled amino
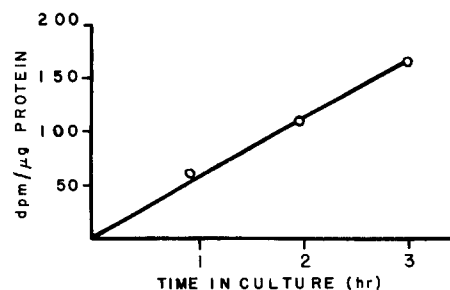

FIGURE 7 Incorporation of *l*-leucine by salivary glands in vitro. Glands were cultured in Schneider's medium containing 4 μc/ml *l*-leucine-^{14}C. At the indicated times three sets of 10 glands each were removed from culture, and protein and radioactivity determinations were done as described under Methods and Materials. Each time point is the average of three determinations.

TABLE III

Thin-Layer Chromatography of an Acid Hydrolysate of Salivary Glands

Radioactivity applied	Radioactivity recovered as lysine	Applied radioactivity recovered as lysine
dpm	*dpm*	*%*
2343	1915	81.7

10 glands were cultured in 0.5 ml Schneider's medium containing 2 μc lysine-^{14}C. After 3 hr incubation, the glands were homogenized in 10% trichloroacetic acid containing unlabeled lysine. The trichloroacetic acid precipitate was hydrolysed, and the hydrolysate was subjected to thin-layer chromatography. The material cochromatographing with authentic lysine was removed and counted.

acid is administered in vivo by injection into larvae (Fig. 9). The gland extract in Fig. 9 was prepared 15 hr after the injection of the labeled amino acid, because incorporation into hemal proteins is maximum at about this time. The same two radioactive peaks are obtained if the extract is prepared 1 hr or 24 hr after the injection of the labeled amino acid. Again, no significant synthesis in vivo of the protein fractions in the R_f range 0.05–1.0 was ever observed. As shown in Table I, the proteins with R_f values in this range are shared by the salivary gland and the hemolymph. If these proteins are synthesized by the salivary gland, they are synthesized at a very slow rate. Actually, it is doubtful whether some of these proteins, such as hemoglobins, would be synthesized in the gland.

TABLE IV

Effect of Puromycin on Lysine-^{14}C Incorporation by Salivary Glands In Vitro

Concentration of puromycin in the medium	Protein	Trichloroacetic acid-soluble radioactivity	Trichloroacetic acid-insoluble radioactivity	Specific activity	Inhibition
$\mu g/ml$	$\mu g/10$ glands	$dpm/10$ glands	$dpm/10$ glands	Trichloroacetic acid-insoluble $dpm/\mu g$ protein	%
0	152	667	62,200	409	0
100	144	1953	2,420	17	96

10 glands were incubated for 10 min in 0.5 ml Schneider's medium containing either no additions or 100 $\mu g/ml$ puromycin. 2 μc lysine-^{14}C then were added to the culture. After 1 hr, the glands were rinsed with Tris-saline buffer and were homogenized in 0.2 ml of 10% trichloroacetic acid containing 1 mg/ml unlabeled lysine. The precipitates then were treated according to the procedure of Siekevitz as described in the text, and the radioactivity was determined in the acid-soluble and acid-insoluble fractions.
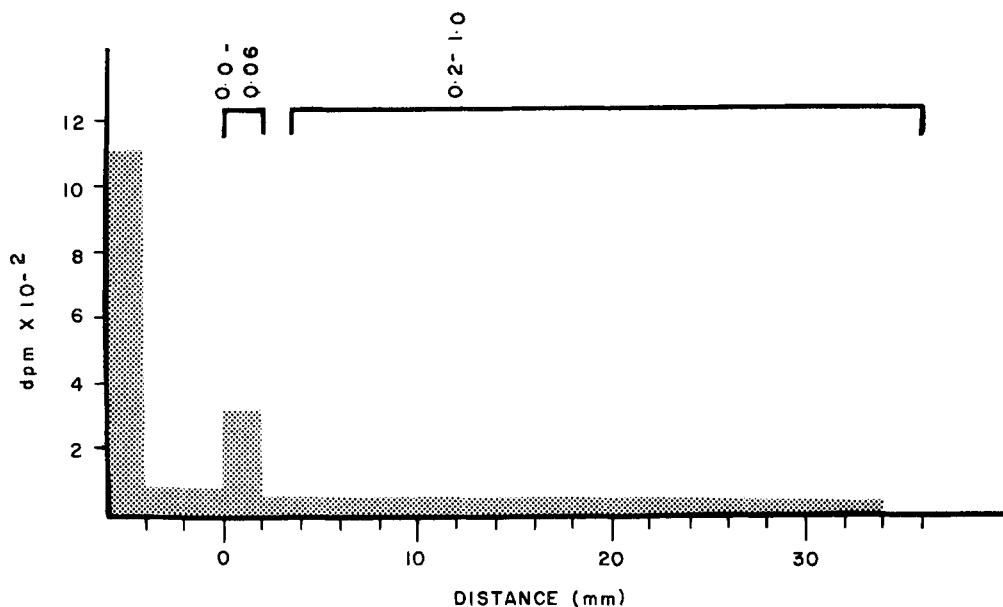

FIGURE 8   Distribution of radioactively labeled proteins following disc electrophoresis of gland extract. Glands were cultured in Schneider's medium containing 4 $\mu c/ml$ of both l-leucine-^{14}C and l-phenylalanine-^{14}C. After 4 hr of incubation, a Tris-saline extract from 40 glands was prepared as described in the text. 250 μg of this extract were applied to the acrylamide column. Electrophoretic conditions and the preparation of the acrylamide columns for counting are described in the text. Brackets indicate the R_f ranges of the separated protein bands. Distance was measured from the cathodic end of the separating gel (0) to the anodic end. Values of less than zero indicate that the radioactivity is present within the spacer gel. About 60% of the added radioactivity can be recovered from the column.

As shown in Figs. 8 and 9, much of the protein synthesized *de novo* in the salivary gland does not freely penetrate the pores of the acrylamide and is present in the spacer gel after electrophoresis. This behavior is not a function of the charge distribution of the labeled proteins, because they are acidic and they should migrate toward the anode during electrophoresis. Most of the labeled protein is present in the supernatant fraction after centrifugation at 105,000 g for 2 hr. How-
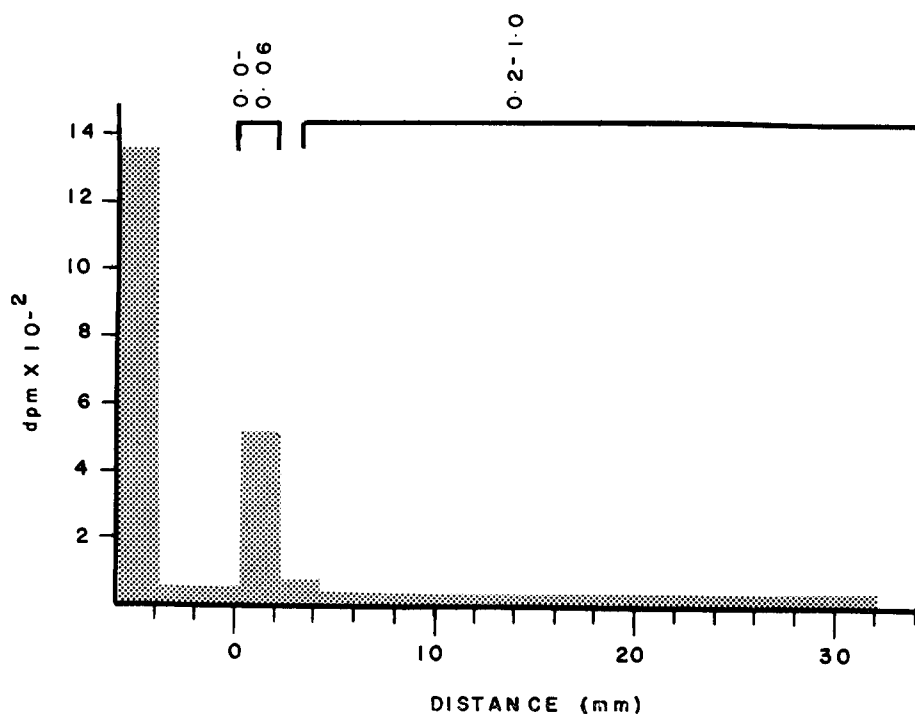
FIGURE 9 Distribution of radioactively labeled gland proteins after disc electrophoresis. Conditions the same as in Fig. 8, except that the Tris-saline extract from 40 glands was prepared 15 hr after the in vivo injection of 0.1 µc lysine-^{14}C per larva.

ever, the labeled proteins tend to aggregate with time and eventually precipitate out of the salivary gland extract. Of the reagents tested (8 M urea, sodium dodecyl sulfate, other detergents, buffers of various ionic strength and pH, sulfhydryl reagents, strong acids and bases, and sodium desoxycholate), only 1.0 N NaOH and sodium desoxycholate were successful in keeping the labeled proteins soluble. When the salivary glands are extracted with 0.2% sodium desoxycholate and the extract is subjected to Bio-Gel A-15m chromatography, the labeled proteins separate as a sharp peak corresponding to an apparent molecular weight of greater than 15,000,000 and a broad peak corresponding to an apparent molecular weight of 200,000 and higher (Fig. 10). The labeled proteins probably are not bound to ribosomes. The same radioactive peaks are obtained in experiments where the labeled precursor is "chased" or diluted with the homologous, unlabeled amino acid. Total hexose was measured to determine if any of this material is mucoprotein. Hexose was not present in significant quantities in any of the fractions

from the Bio-Gel column. The proteins synthesized in the gland appear to be both polydisperse and in a highly aggregated state, and this latter property probably accounts for their slow migration during disc electrophoresis.

As demonstrated by radioautography, most of the proteins synthesized in the salivary gland are secreted from it. Before these results are presented, it will be helpful to consider briefly the morphology of the salivary gland. More comprehensive descriptions can be found in references 3, 8, and 14. A diagrammatic representation of a fourth instar salivary gland is presented in Fig. 11. Essentially three cell types form the entire larval gland. The secretory duct and the region at the base of the duct are composed of many small squamous cells. The chromosomes of these cells vary in degree of polyteny, but they are always much less polytenic than the chromosomes of the 30 or so large cells which form most of the gland. There are also two cells covering the anterior portion of the gland lumen. These two cells can be distinguished from the other cells of the gland by their flat shape, by their large size, and by the
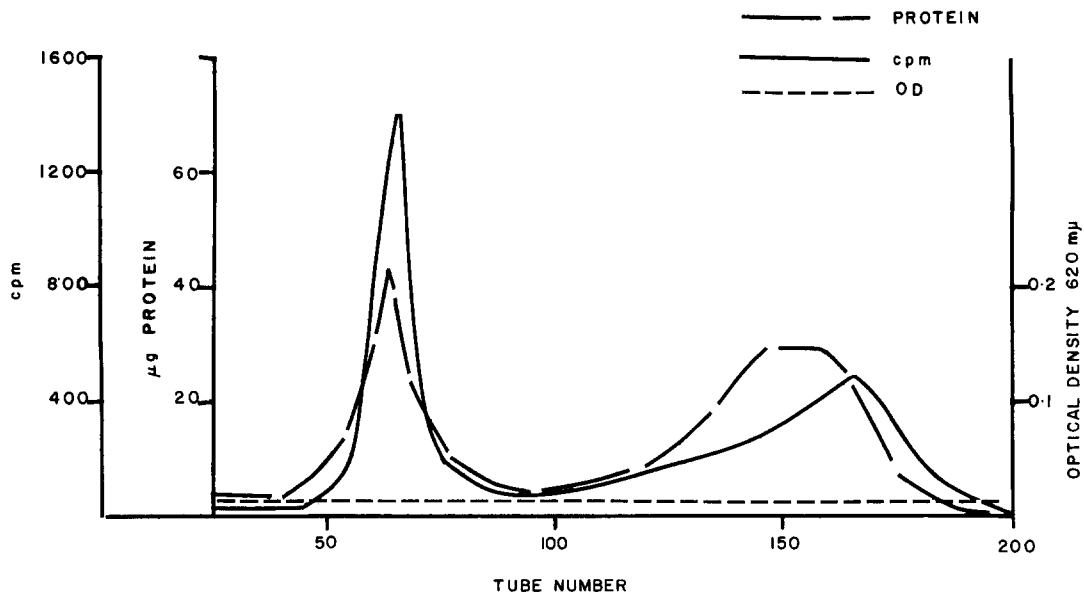
FIGURE 10 Molecular sieve chromatography of a desoxycholate extract of salivary glands. Groups of 10 glands were exposed in vitro to 4 μc/ml leucine-^{14}C for 1.5 hr. 60 such glands then were mixed with 100 glands that had not been exposed to the labeled amino acid. The preparation of the 0.2% sodium desoxycholate extract and the Bio-Gel A 15m column is described in the text. The column was developed with 0.1 M NaCl containing 0.2% sodium desoxycholate. 1.0 ml of each 1.5 ml fraction was added to 10 ml of Bray's solution for counting. The remainder was used for the determination of total hexose (absorption at 620 mμ) and protein.

fact that their chromosomes are intermediate in degree of polyteny. It is also of interest that these two cells, in contrast to the large gland cells, do not appear to have a well-developed rough endo-plasmic reticulum when examined with the electron microscope (14). The lumen extends through-out the gland and protrudes among cells.

Salivary glands in vitro were given a 15 min pulse of lysine-^{3}H; a 100-fold excess of unlabeled lysine then was added to the culture, and at intervals thereafter the glands were prepared for radioautography. The results of a typical experiment are summarized in Fig. 12. At least 80% of the protein synthesized during the pulse is secreted into the gland lumen during the 4 hr chase with unlabeled lysine. In some experiments, as much as 90% of the protein synthesized is eventually secreted from the gland cells. When the labeled amino acid is administered in vivo, labeled protein is eventually (15 hr) secreted from the larvae.

The synthesis of secretion proteins is confined mainly to the 30 large gland cells containing the

giant chromosomes. The small cells forming both the base of the duct and the duct do not appear to synthesize any secretion proteins. The two cells covering the anterior portion of the gland lumen are not very active in total protein synthesis; if they secrete any protein, the amount is certainly much less than that in the large cells.

It is important to emphasize that the 10 protein fractions shared by the salivary gland and the hemolymph (R_f 0.05–1.0, see Table I) probably are not synthesized in the salivary gland (Figs. 8 and 9). These proteins are synthesized in other larval tissues, and they are synthesized whether the salivary gland is present or absent. Figs. 13 and 14 show the distribution of incorporated radioactivity following disc electrophoresis of hemolymph collected from two groups of larvae. In one group, the salivary glands were isolated from the hemocoel before injection of amino acids-^{14}C into the isolated portion of the hemocoel; the salivary glands were not isolated from the hemocoel in the second group of larvae. The patterns of labeled hemal proteins resolved from
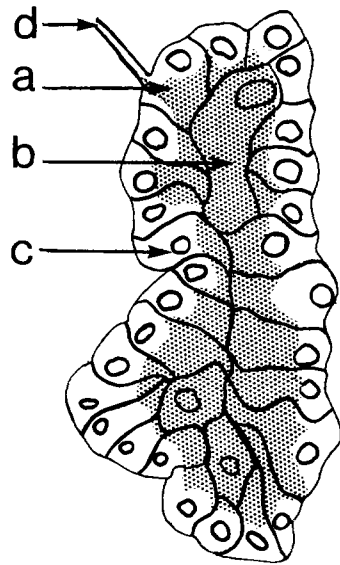
FIGURE 11 Diagrammatic representation of a salivary gland from a fourth instar *Chironomus tentans. a* denotes the region at the base of the duct composed of many small squamous-like cells; *b* denotes one of the two larger cells, intermediate in degree of polyteny, covering the anterior portion of the gland lumen; *c* denotes one of the 30 large cells containing highly polytenic chromosomes and forming most of the gland; *d* denotes the secretory duct. About × 250.

the two groups of larvae are essentially identical, showing that the major hemal proteins are synthesized even in the absence of the salivary gland.

As shown previously (Fig. 6, Table II), at least some of the proteins shared by the salivary gland and the hemolymph are present also in the secretion, and they can be synthesized independently of the salivary gland. They do not appear to be synthesized by the gland. We conclude that these proteins are synthesized in larval tissues other than the salivary gland and are released into the hemolymph. The salivary gland then sequesters them from the hemolymph and subsequently secretes them.

It should be mentioned that the proteins shared by the salivary gland and the hemolymph (R_f 0.05–1.0, see Table I) are present in the hemolymph as labeled proteins after the in vivo injection of amino acids-^{14}C (Fig. 14). They do not contribute to the radioactivity profile of the gland extract in Fig. 9 because they are diluted with their unlabeled homologues already existing in the hemolymph. Therefore, because of the low

specific radioactivity of the proteins in the hemolymph, the unlabeled hemal proteins have a better chance of being taken up by the gland during the course of the experiment depicted in Fig. 9 than do the equivalent labeled proteins.

DISCUSSION

The results presented in this paper are consistent with a mechanism of secretion formation involving both the *de novo* synthesis of secretion proteins and the uptake, transport, and secretion of hemal proteins by the salivary gland. Uptake of hemal proteins appears to be selective, assuming that the proteins in extracts reflect those in the intact salivary gland. Thus, some hemal proteins are concentrated by the salivary gland (Fig. 2), while others are completely excluded (Table I) from the gland. Selective uptake of proteins has been observed in other tissues, notably the insect oocyte (29, 33, 34). Although the cell basement membrane and the cell surface have been suggested (29, 34) as sites where selection might occur, the *modus operandi* for selective uptake of proteins is not known.

Previous experiments from this laboratory have shown that radioiodinated human serum albumin is taken up by the salivary gland and is secreted intact (9, 18). Experiments with this labeled protein suggest that some differentiation of function exists in the cells forming the gland. The 30 or so large cells containing the giant chromosomes are most active in the synthesis of secretion proteins. These cells have a well-developed rough endoplasmic reticulum and Golgi apparatus, and electron microscopic radioautography shows that the labeled protein is present in the endoplasmic reticulum, in the Golgi apparatus, and eventuallly within secretion granules at the luminal border (14). The 30 large cells also sequester serum albumin from the hemolymph, but only part of the sequestered protein is secreted. On the other hand, the cells forming the duct and the base of the duct appear to synthesize very little if any secretion protein, but these cells are most active in the uptake and transport of both human serum albumin and ferritin (14). The two cells covering the anterior portion of the gland lumen also appear to be more active in the uptake and transport of proteins than in the synthesis of secretion proteins. The absence of a well-developed rough endoplasmic reticulum (14) in these two cells also
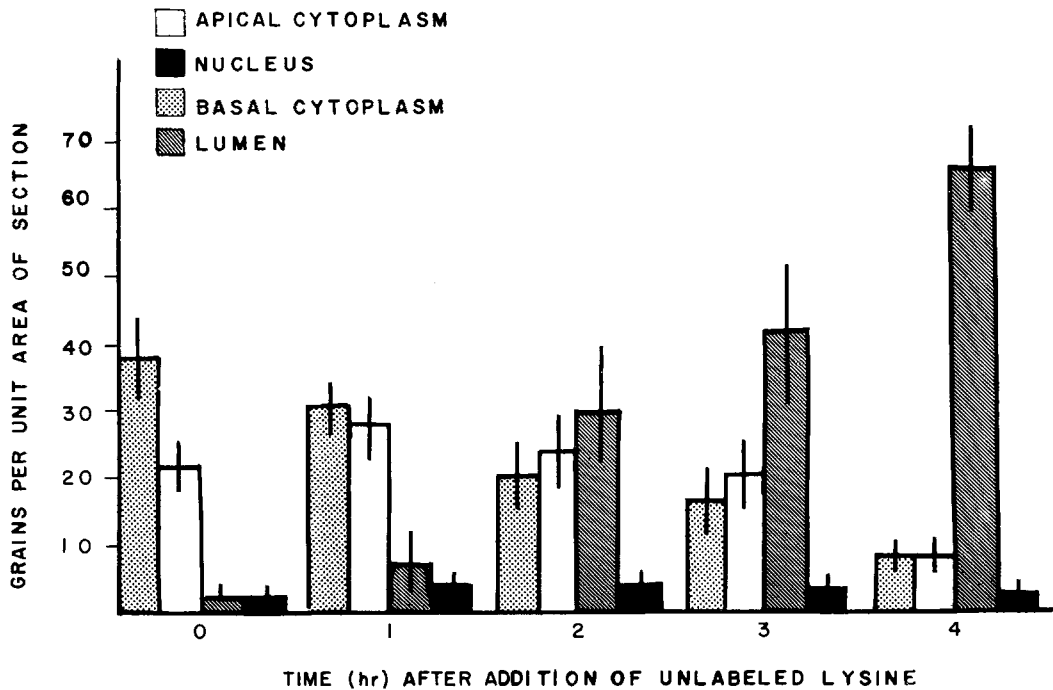
FIGURE 12   Distribution of radioautographic grains over salivary gland cell components. Glands in vitro were exposed to lysine-^{3}H for 15 min before addition of excess unlabeled lysine. At the indicated times, the glands were prepared for radioautography as described in the text. Grain counts were done only over the 30 large cells. Grains were counted over four areas of the section: the cytoplasm immediately adjacent to the hemal border of the cell (basal cytoplasm); the cytoplasm adjacent to the gland lumen (apical cytoplasm); the gland lumen; and the nucleus. 30 sections, taken at random, were examined per gland. A unit area was 23 μ^2, and 10 areas were counted in each of the four locations within the section. Five glands were examined. The bars represent the standard deviations of the means among the five glands.

suggests that they are not involved primarily in the synthesis of secretion proteins.

There is evidence suggesting that uptake and transport contribute to the salivary secretion in still other dipteran species. For example, many of the secretion antigens in *Drosophila virilis* (27) and in *Bradysia* (28) are not unique to the secretion and are distributed in other larval tissues. It remains to be seen whether the salivary gland synthesizes or sequesters and transports these antigens.

Of interest also are the relevant studies of Baudisch (1) and Mechelke (22) who demonstrated the relationship of specific chromosomal Balbiani rings to the presence of carotenoids in the anterior lobe of the salivary gland of *Acricotopus lucidus*. Since carotenoids are not known to be synthesized by insects, this secretion product may well be derived by uptake and transport, and

uptake may be controlled by specific chromosomal loci in this organism.

The proteins synthesized in the salivary gland of *Chironomus tentans* are highly aggregated. Much of this protein has an apparent molecular weight of greater than 15,000,000. Both the salivary gland and the secretion show a strong periodic acid-Schiff reaction (2, 12), and the gland actively incorporates and secretes radioactive sulfate (13). These results suggest that mucoproteins are synthesized and secreted by the salivary gland. However, recent experiments of H. Laufer and S. Tamamura (1967. Unpublished results.) indicate that the secretion contains less than 4% carbohydrate. Evidence presented in this paper also indicates that the proteins synthesized in the gland contain little total hexose. If the carbohydrate moiety represents 1% or less of the weight of the protein molecule, the protein can still be
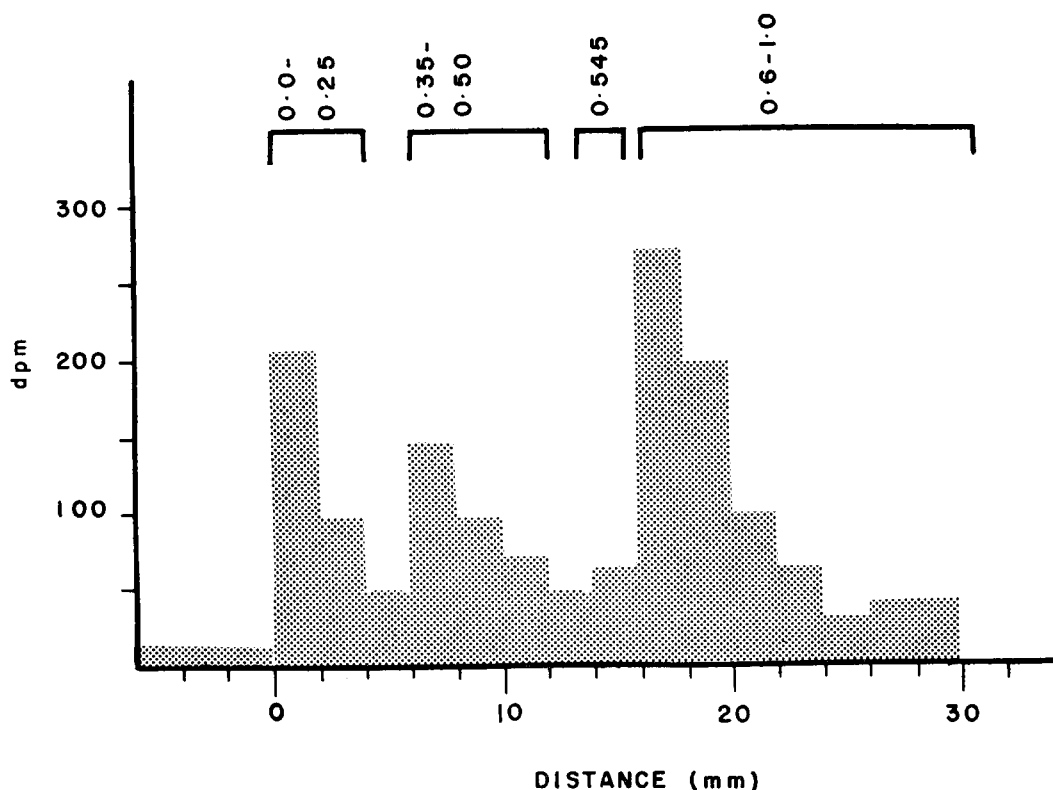
FIGURE 13 Distribution of radioactivity following disc electrophoresis of hemolymph from ligatured larvae. Larvae were ligatured to isolate the salivary glands from the hemocoel as described in the text and in the legend to Table II. Each larva then was injected with 0.1 μc of mixed l-amino acids-^{14}C. 15 hr later, the hemolymph was collected, pooled, and dialyzed against 0.1 M NaCl. 300 μg of hemal protein were applied to the acrylamide column. The columns were prepared for counting as described in the text and in the legend to Fig. 8. Brackets indicate the R_f ranges of the separated protein fractions. Approximately 80% of the added radioactivity can be recovered from the column.

classified as a mucoprotein. The colorimetric technique used here to assay total hexose is not sufficiently sensitive to detect small amounts of carbohydrate, and more sensitive techniques may yet show that mucoproteins are synthesized and secreted by the salivary gland.

Laufer and Tamamura did find significant amounts of lipid in the secretion, and lipids are also periodic acid-Schiff positive (25). Since an agent known to react with lipids, sodium desoxycholate, will "solubilize" the secretion proteins synthesized by the gland, these proteins may be conjugated to lipid. It is also possible that the proteins synthesized by the gland are covalently cross-linked to form a giant three-dimensional protein molecule, rich in hydrophobic groups, which functions as a matrix for the soluble pro-

teins of the secretion. Since the proteins synthesized by the gland are for the most part undefined, experiments are currently in progress to determine their exact chemical nature.

It may be more than fortuitous that both the tissue-specific salivary gland antigens and the proteins synthesized by the gland behave in the same manner during disc electrophoresis (compare Figs. 5, 8, and 9). It is conceivable that the proteins synthesized in the salivary gland and secreted from it are identical with the gland-specific antigens. These antigens can not be detected in the secretion, but there are physical-chemical changes in the secretion both upon release from the gland cells (14) and upon transfer from the gland lumen to the external environment. The material within the lumen is a clear,
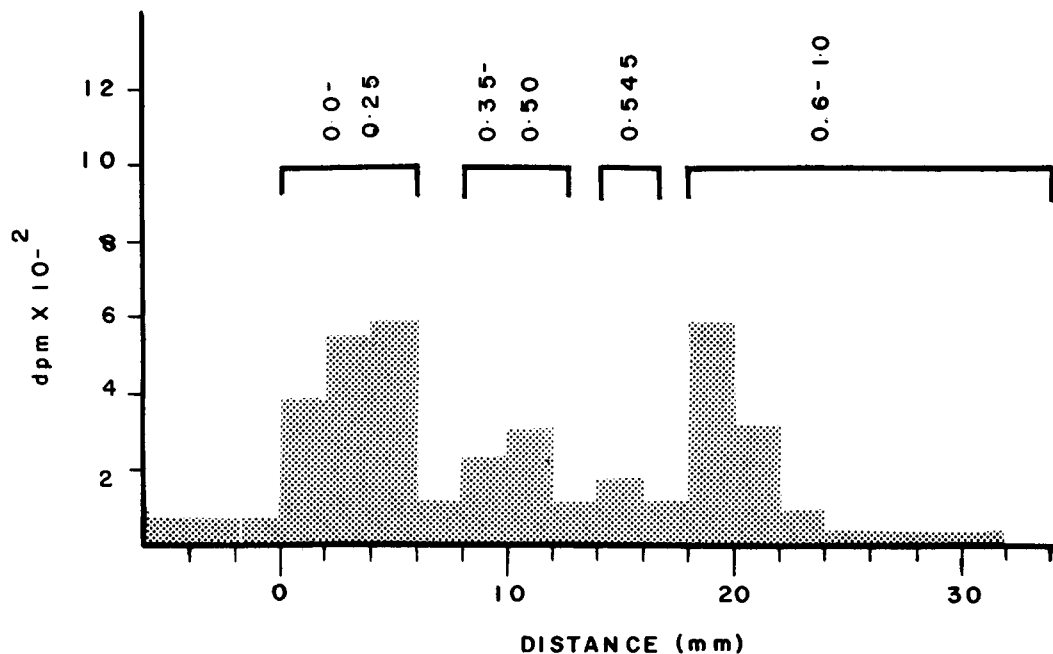
FIGURE 14 Distribution of radioactivity following disc electrophoresis of hemolymph. Conditions the same as in Fig. 13, except that the salivary glands were not isolated from the hemocoel, and 800 μg of protein were applied to the acrylamide column.

viscous solution which, when released into the environment, becomes white, elastic, and fibrous. The secretion in this form is insoluble in most reagents. This property makes the secretion extremely difficult to analyse. The apparent absence of gland-specific antigens in the secretion may be a function of the relative insolubility of the secretion proteins.

Finally, the relationship of puffing at specific chromosomal loci to the formation of the secretion by *de novo* synthesis and by uptake and transport of proteins has been examined. These experiments have been published in preliminary form (9) and will be presented in detail in a subsequent paper. Briefly, the results show that the synthesis of secretion proteins occurs on relatively stable messenger ribonucleic acid templates. Chromosomal RNA synthesis can be completely inhibited for 12 hr and longer by actinomycin D without affecting salivary gland protein synthesis. While the synthesis of secretion proteins is completely inhibited by puromycin, uptake and transport are relatively resistant to the effects of this antibiotic. Uptake and transport of proteins continue in the absence of both gland protein synthesis and gland ribonucleic acid synthesis. These results suggest that the gland proteins involved in the transport of secretion do not turn over rapidly.

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