INTRODUCTION

The salivary glands of Diptera are organs homologous to the silk glands of Lepidoptera, in that both are labial glands (1). Larvae of several Chironomus species produce a salivary secretion which, according to its properties (2), fits the silk definition given by Rudall and Kenchington (3). Formation of Diptera salivary secretion has been investigated in some species of Chironomus (4, 5, 6). The results obtained indicate that the proteins present in the salivary secretion are in part synthesized by the salivary gland and in part sequestered from the hemolymph. However, this conclusion is not fully accepted (7).

We have studied the origin of the salivary secretion proteins of Rhynchosciara americana larvae. These larvae produce a salivary secretion that is used in spinning a communal cocoon, inside of which the fourth instar larvae undergoes pupation and where development goes on until the emergence of the imago. The R. americana communal cocoon is made of threads and plaques of a highly insoluble material which shows characteristics of a true silk.

METHODS

Animals

Experiments were all performed with fourth instar female larvae. The fourth instar was divided into six periods, characterized by physiological and morphological events. The division adopted here is a slight modification of that proposed by Guaraciaba and Toledo (9), made by Terra et al. (20). The larvae used were at the fourth period (54 days old) or at the second period (45 days old). The animals were raised in the laboratory as previously described (10).

Drug and Isotope Administration

Drugs and isotopes were injected into the larval hemocoel, using needles prepared from microcaps of 15 µl (Drummond Scientific Co., Broomall, Pa.).

Before the injections, the larvae were anesthetized

\footnotetext{Rhynchosciara americana (Wiedemann, 1821) (Diptera, Sciaridae) was redescribed as angelae (Nonato and Pavan, 1951), and their synonymy was demonstrated by Breuer (8).}
with ethyl ether. Each larva was injected with 2 µl of [4,5-3H]leucine (40 Ci/mM; 0.5 mCi/ml) purchased from Schwarz BioResearch Inc., Orangeburg, N. Y. In some experiments the larvae were also injected with 2 µl of a 5 µg/ml puromycin solution.

**Preparation of Extracts**

After the desired incorporation times, the larvae were placed on crushed ice and when they become quiescent their posterior ends were punctured. The hemolymph drops thus produced were collected into a centrifuge tube maintained at 0°C and were centrifuged, at 0°C, for 5 min at 5,000 g, for hemocyte sedimentation. The supernatant of centrifugation was diluted conveniently with 0.1 M NaCl.

After the hemolymph withdrawal, the larvae were dissected under a stereoscopic microscope and the salivary glands were collected and rinsed in physiological solution for *Rhynchosciara* (11); the salivary glands were homogenized with 0.1 N NaOH in a Potter-Elvehjem homogenizer (Potter Instrument Co., Inc., Melville, N. Y.). The homogenate was centrifuged at 12,000 g for 10 min, at 0°C.

The obtaining of salivary secretion and of intracellular proteins from the same salivary gland was carried out as follows. The dissected glands were placed into small centrifuge tubes, and 0.5 ml of 0.1 M carbonate-bicarbonate buffer, pH 9.5, was added. The salivary glands were then broken into large pieces with a needle. The salivary secretion stored in the lumens solubilized in the buffer (R. Meneghini, personal communication) and was then separated from the broken glands by centrifugation at 2,000 g for 5 min. The broken glands were then homogenized with 0.1 N NaOH in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 g for 10 min, at 0°C, and the supernatant corresponded to an intracellular protein preparation.

Protein in the extracts was determined by the method of Lowry et al. (12) as modified by Oyama and Eagle (13).

**Counting Procedures**

Each extract, prepared as described above, was treated according to the method described by Mans and Novelli (14) for protein purification and radioactivity counting. Radioactivity was counted in a liquid scintillation spectrometer LS-100 (Beckman Instruments, Inc., Fullerton, Calif.) with an efficiency of 45%. The scintillation solution used contained 4 g of 2,5-diphenyloxazol and 0.1 g of 1,5-bis-2-(5-phenyloxazolyl) benzene per liter of toluene.

For the determinations of acid-soluble radioactivity in the hemolymph, trichloroacetic acid was added to 0.1 ml hemolymph samples to achieve a final concentration of 8% wt/vol at 0°C. The suspension obtained was then centrifuged for 10 min at 12,000 g and the radioactivity in the supernatant was counted by pouring the supernatant into 10 ml of Bray solution (15) in the spectrometer as specified above.

**RESULTS**

**Kinetics of [4,5-3H]Leucine Incorporation**

Kinetic studies of the incorporation of [4,5-3H] leucine into salivary gland and hemolymph proteins gave the results shown in Fig. 1. In this figure the data on hemolymph acid-soluble radioactivity are also included. This last measurement gives information about the amount of radioactive precursor remaining in the animal hemolymph as a function of time. The increase observed between 5 and 6 h of incorporation in this fraction must be a consequence of an uneven recuperation of the hemolymph TCA-soluble supernatant.

Hemolymph protein labeling shows an initial lag, increases linearly for 6 h, and finally attains a constant value. Salivary gland protein labeling increases linearly until a maximal value is attained, then it decreases and then increases again until a new maximal value is attained, after which a linear fall takes place. It is interesting to note that the second increase in specific activity of the salivary gland proteins occurs when the level of labeled amino acid in the hemolymph is small in relation to the initial value. The existence of two peaks of labeling in salivary gland proteins was also observed in another independent experiment shown in Fig. 3. A tentative explanation for the appearance of the second peak of specific activity in salivary gland proteins could be the occurrence of a sequestration of hemolymph proteins.

**Effect of Puromycin in [4,5-3H] Leucine Incorporation**

In order to test the validity of the above hypothesis, we have done an experiment similar to that initially described but in which the larvae were injected with puromycin at the moment in which the first maximal value of gland protein specific activity was attained. In a control experiment, we verified that the amount of puromycin used causes, when injected before [3H]leucine, a 96% inhibition of the labeling of gland protein and a 94% inhibition in the labeling of hemolymph proteins.
The results of such an experiment are shown in Fig. 2. One sees that the specific activity of the hemolymph proteins, which in the experiment described in Fig. 1 attained a constant value, in this new experiment decreases continuously, after rising to a maximum. The second increase of gland protein specific activity is observed again, as in Fig. 1. Since this increase occurs in the presence of puromycin, it cannot be due to protein synthesis in salivary glands. This increase is explained if we assume that there is a sequestration of hemolymph proteins, as proposed before.

The difference of timing between the counts in gland proteins in Fig. 1 and in Fig. 2 could be a consequence of the utilization of larvae originating from different laying groups in the two experiments. The larvae of R. americana originating in the same egg clutch are very much alike and are of the same sex, while larvae of a different egg clutch, even of the same chronological age, may differ significantly in physiological responses (9).

**Labeled Protein Eliminated with the Salivary Secretion**

If the above interpretation is correct, one would expect that the secreted proteins of [4,5-3H]leucine-injected larvae would show two cycles of specific activity increase. The secretion eliminated...
during the first specific activity increase would be rich in labeled proteins synthesized by the salivary glands whereas that of the second cycle of increase would be rich in labeled proteins sequestered from the hemolymph.

In order to verify the possible occurrence of these two cycles of specific activity increase of the salivary secretion proteins, an experiment was carried out in which we tried to follow the elimination of radioactivity associated with salivary secretion proteins eliminated by second period larvae injected with $^3$H]leucine; and, at the same time, we determined the radioactivity incorporated into the proteins remaining in the salivary gland cells. Larvae of the second period were used because they have a larger amount of salivary secretion stored in their glands than those used in the experiments previously described, that is, fourth period larvae (unpublished results). The results of this experiment are shown in Fig. 3. It can be seen that the secreted proteins show two cycles of specific activity increase in accordance with our expectation.

The second peak of incorporated radioactivity
FIGURE 3  Labeling of salivary secretion proteins by [4,5-3H]-leucine. For each determination, five larvae were injected with 2 µl of [4,5-3H]leucine. After the incorporation times indicated, the hemolymph was collected and the salivary glands were dissected and rinsed in 0.1 M NaCl. The salivary secretion and the glands free from secretion were then prepared as described in Methods. The specific activity of hemolymph proteins and acid-soluble radioactivity in the hemolymph were determined as in Fig. 1. Since the protein content of the salivary secretion obtained was too low to be determined by the usual methods, the specific activity of the secreted proteins was calculated by dividing the incorporated radioactivity by the total protein content of the salivary glands from which it was obtained.

The difference of the profiles of protein counts in glands between Fig. 1 and Fig. 3 is a consequence of the fact that in the profile of Fig. 1 the salivary secretion is included while in the profile of Fig. 3 it is not. However, a direct comparison can be made between the profile of protein counts in glands in Fig. 1 and the sum of the two profiles in Fig. 3.

DISCUSSION

The data presented indicate that salivary secretion proteins of *R. americana* have a double origin. The first specific increase (Fig. 1) indicates protein synthesis in the salivary gland, and, since a similar increase of specific activity occurs also in secreted proteins (Fig. 3), we concluded that some proteins synthesized in salivary gland are secreted. The second increase of specific activity
(Figs. 1, 2, and 3) is a consequence of hemolymph protein sequestration by the salivary glands and its transference into salivary secretion without previous hydrolysis.

The capture of hemolymph proteins by some organs of several insect orders has been demonstrated by immunological techniques (16), autoradiography (17), and by electron microscopy (18). Therefore, our observation that R. americana salivary glands sequester proteins from the hemolymph does not constitute an isolated case.

The amino acid composition of the salivary secretion of R. americana and that of the insoluble proteins from the cocoon are similar (19). This result indicates that the majority of the salivary proteins from the cocoon are similar (19). Therefore, our observation that the silk proteins of R. americana which are synthesized ex-clusively from hemolymph free amino acids, in contrast with Bombyx mori silk which is synthesized exclusively from hemolymph free amino acids (21).

The results discussed here, though not yet conclusive, lead to the working hypothesis that the nematoceran Diptera synthesize silk in a peculiar way involving sequestration of hemolymph proteins.

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