DEVELOPMENTAL STUDIES OF THE DIPTERAN SALIVARY GLAND

II. DNase Activity in *Chironomus thummi*

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ABSTRACT

The deoxyribonuclease (DNase) activity of the dipteran (*Chironomus thummi*) salivary gland, measured both enzymatically and immunochemically, increases about 7-fold with the onset of metamorphosis. The increase in DNase activity occurs at a time when the activities of other enzymes and the total protein content are decreasing. The increased DNase activity is followed by glandular destruction. It is suggested that the alterations of this activity may be regulated by the activities of specific chromosomal sites, and that the enzyme may, at least in part, account for the glandular destruction observed at the time of increased enzyme activity.

INTRODUCTION

One example of nucleo-cytoplasmic interaction occurring during salivary gland development is the effect of the insect molting hormone, ecdysone, on chromosomal differentiation (Clever, 1961). The hormone alters the chromosomal puffing pattern to one normally seen only later in the course of development. A second type of interaction is necessitated by present concepts of protein synthesis in the cytoplasm following as a consequence of chromosomal puffing. This in turn may affect cellular differentiation. In order to learn more about nucleo-cytoplasmic interactions which occur during development we are investigating developmental changes in protein as correlated with chromosomal differentiation in the salivary gland of *Chironomus thummi*.

The aim of the present series of reports is to characterize the kinds of proteins present in the gland, to describe the changes they undergo, and to determine how these alterations relate to chromosomal activity during development. Particular attention was directed to enzyme activities in the cytoplasm. Until now, the paucity of suitable cytological and biochemical "markers" with which chromosomal activities can be correlated and ultimately identified has posed a serious problem.

Enzyme activities of the *Chironomus* salivary gland which we have reported elsewhere include those of trehalase, hyaluronidase, malate dehydrogenase, and protease. The changing titer of these enzymes was correlated with the changing morphology of certain chromosomal loci (Laufer, 1963; Laufer, Nakase, and Vanderberg, 1964). These enzyme activities increased at rates proportional to the increase in amount of total glandular protein during prepupation, only to decrease as the gland degenerated in the pupal stage (Laufer, 1963; Laufer, Nakase, and Vanderberg, 1963 *a*). Of particular interest and significance was the observation that hyaluronidase, trehalase, and protease activity occur in the secretion (Laufer and Nakase, 1965). The loci whose activity patterns most closely paralleled salivary gland enzyme levels were the large Balbiani rings of regions *b* and *c* of chromosome 4

(nomenclature of Keyl, 1957) and the nucleolus (Vogt-Köhne, 1961; Laufer, 1963). Other loci at which puffs increased in size during late larval life and became inactive during prepupation have been described by Kroeger (1963). Thus the enzymatic content of the gland appears to be associated with the secretory process and with actively puffed regions of chromosomes.

The present report is concerned with the enzyme deoxyribonuclease (DNase), which is low in activity in the larval salivary gland. However, later, in the prepupal stage, just before the metamorphic molt, total enzyme activity of DNase per gland as well as its specific activity (per total protein) increases significantly. Therefore the pattern of DNase specific activity is different from that of the other enzymes studied in the gland. Changes in this enzyme coincide with the changing activities of chromosomal puffs which are activated and inactivated only at critical developmental stages. DNase determinations revealed the possibility that the enzyme plays a significant developmental role. Preliminary reports concerning these findings were presented by Laufer, Nakase, and Vanderberg (1963 *a* and *c*).

METHODS AND MATERIALS

Chironomus thummi were laboratory raised in plastic tanks in aerated tap water at a temperature of about 20°C. They were fed precooked nettle powder (Penick & Company, New York) three times weekly. The larvae were classified, according to their appearance, into 4 developmental categories: mid-4th instar larvae, late-4th instar larvae, early prepupae, and late prepupae (Laufer, Nakase, and Vanderberg, 1964). Salivary glands dissected from these larvae were rinsed 3 to 5 times in 0.1 M NaCl to wash off body fluid. Three washes were sufficient to remove contaminating proteins; *i.e.*, to bring them to constant activity. The washed glands were assayed for protein content and for enzyme activity. Each set of five pairs of salivary glands was homogenized in 0.2 ml of 0.2 M acetate buffer at pH 7.0 in all-glass microhomogenizers. DNase assays were performed on the supernatant of salivary gland homogenates after centrifugation at 5,000 *g* for 10 minutes. An optimal reaction mixture was determined from among several buffers and a range of pH, substrate, and enzyme concentrations. The reaction mixture found to give the most satisfactory assay included 0.2 ml of 0.2 M acetate buffer, pH 7.0; 0.2 ml of polymerized DNA (Sigma Chemical Co., St. Louis, Missouri) as substrate (2 mg/ml); 0.1 ml of 0.1 M $MgSO_4$; and 0.2 ml of salivary gland extract from 5 pairs of glands. In this mixture the reaction rate was proportional to the amount of homogenate added and to the incubation time. The reaction was stopped after 120 minutes of incubation at 37°C by the addition of 0.2 ml of 3 M TCA. The precipitate was sedimented at 5,000 *g* for 10 minutes. To 0.5 ml of supernatant 1.0 ml of diphenylamine reagent (Dische, 1930) was added. The color that developed after 20 minutes at 100°C was read at 600 mμ on a Beckman DU spectrophotometer. Protein contents of salivary glands were determined by the procedure of Lowry *et al.* (1951) with the Folin-Ciocalteu reagent.

The DNase activity of the salivary gland was also investigated by immunochemical procedures. Extracts containing the enzyme activity produced a precipitate when reacted with immune serum from rabbits immunized with crystalline beef pancreatic DNase I.[1] Quantitative antibody precipitations were performed according to the micromethod described by Kabat and Mayer (1961). Protein determinations were performed by the method of Lowry *et al.* (1951). 0.1 ml of immune serum–precipitated crystalline beef DNase I (Sigma Chemical Company) in increasing quantities in the range of 0.5 to 50 μg of enzyme protein. Agar diffusion test in Ouchterlony plates produced one heavy sharp line of precipitation when DNase antisera were reacted with pancreatic DNase I. Likewise, only one line, though a less copious precipitate, appeared when this anti-serum reacted with dipteran antigen.

RESULTS

The larval salivary gland of *Chironomus thummi* undergoes a normal cycle of rapid growth in the last larval instar. This growth is followed by a decrease in mass, and ultimately by degeneration during metamorphosis. Our studies of total protein content and DNase activity during this 4th larval stage of *Chironomus* show several significant alterations. The protein content of the gland increases in the 4th larval instar until the stage of prepupation when it decreases (Table I). While the protein content is increasing, in phases of rapid enlargement of the mid- and late-4th instar larval gland, the total DNase activity decreases somewhat, and falls to almost undetectable levels. With approaching metamorphosis this trend is reversed; a substantial increase in DNase activity

[1] We thank Dr. Herbert Rosenkranz and Dr. Sam Beiser for their generous gift of some of the immune sera used in these experiments. These sera also precipitated other DNase preparations such as bovine DNase II and DNase from *Streptococcus* but did not react with other proteins such as serum albumin (Rosenkranz, personal communication; Laufer and Nakase, unpublished data).

TABLE I

Protein Content and DNase Activity of Chironomus thummi Salivary Glands at 4 Different Stages of Development

Stage	Number of determinations	Protein content (μg/pair of glands)		Number of determinations	DNase activity (OD/5 pairs of glands)		Specific activity (activity/mg protein)
		SD	SE		SD	SE	
1. Mid-4th instar	22	9.3 ± 3.16*	0.67	16	0.016 ± 0.018‡	0.005	1.72
2. Late 4th instar	26	15.8 ± 4.58*	0.88	16	0.007 ± 0.010§	0.003	0.449
3. Early prepupa	25	24.7 ± 6.78*	1.36	16	0.027 ± 0.021‖	0.005	1.09
4. Late prepupa	26	16.2 ± 6.00*	1.18	16	0.048 ± 0.023¶	0.006	2.98

* Statistical analysis by the t test revealed that the protein content at every stage of salivary gland development is significantly different from that of every other stage. P is less than 1 per cent.

‡ DNase activity is significantly different at the 1 per cent level by the t test when mid-4th instar and late prepupae are compared.

§ DNase activity is significantly different at the 1 per cent level by the t test when late 4th instars are compared to either early prepupae or late prepupae.

‖ DNase activity is significantly different at the 1 per cent level by the t test when early prepupae are compared with late 4th instar. DNase activity of early prepupae is possibly significantly different at the 5 per cent level, when compared with the late prepupa.

¶ DNase activity is significantly different at the 1 per cent level by the t test when late prepupae are compared to mid-4th instar and late 4th instar. DNase activity of late prepupae is possibly significantly different at the 5 per cent level when compared to the early prepupa.

is observed as the gland progresses from late-4th instar to the early prepupa and on to the late prepupa (Table I). The greatest rise in total DNase activity and specific activity of the homogenate (activity/mg protein) occurs in the late prepupa, at a time when the *total* protein in the gland is actually *decreasing*. The total increase of DNase activity, from the lowest to the highest value, is approximately 7-fold. These are significant changes in enzyme activity during development despite considerable variations in each group of determinations. Table I also indicates the magnitude of the standard error of the mean. Replicate samples from pooled homogenates, when assayed separately for DNase activity, gave highly reproducible results. This fact suggests that the variation obtained for individual determinations is due to physiological differences among the various batches of animals.

The results of a statistical analysis comparing DNase activities of glands at four different developmental stages are included in Table I. The activity in the late prepupa is highest and is also significantly different from the levels of activity found at the earlier stages. This observation substantiates the major contention that there is a quantitative increase in *amounts* of DNase in this final stage of glandular development when the level of other enzymes and of total protein decreases.

The increased DNase activity found at the end of larval life, in the late prepupa, is marked. Virtually no activity was detectable in 50 per cent of the mid-4th instar (stage 1) glands, and 80 per cent of those from late-4th instar larvae (stage 2). In contrast, all late-prepupal stage glands (stage 4) were found to have DNase activity.

The ability of *Chironomus* salivary gland extracts to combine with beef pancreas DNase antibodies permitted a quantitative estimation of salivary gland DNase during the larval-pupal transition. Such immunochemical determinations depend upon the antigenic properties of the protein rather than its enzymic activity and therefore constitute an independent assay for the protein. A plot of the precipitable protein present after DNase antibodies had reacted with salivary gland homogenates of different stages shows a quantitative increase with increasing developmental age (Fig. 1). From 1.2 μg to 24 μg antigen-antibody complex is precipitable for 10 pairs of salivary glands. The amount of antigen precipitated depends on the stage and increases about 7-fold during development. The precipitated protein is equivalent to a

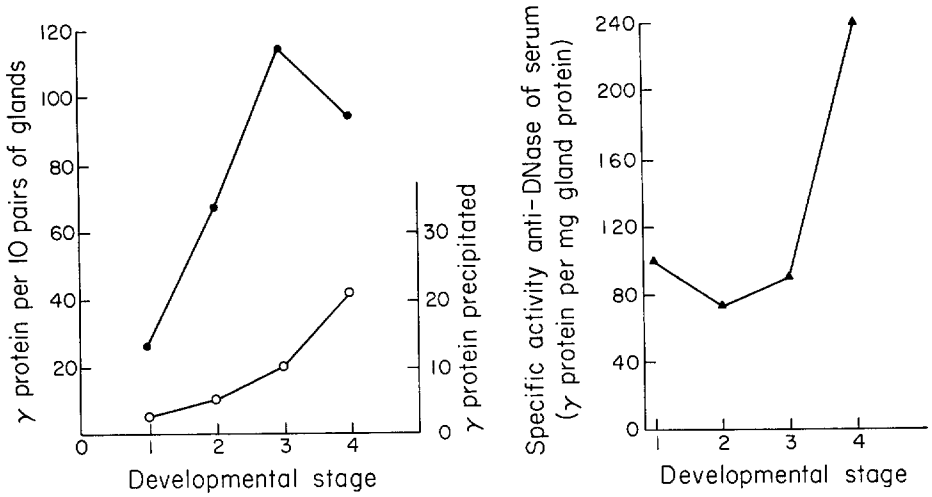

FIGURE 1 Quantitative precipitation of salivary gland DNase by anti-DNase. Developmental stages are: *1*, mid-4th instar; *2*, late-4th instar; *3*, early prepupa; *4*, late prepupa. The figure on the left indicates the existence of a seven-fold increase in DNase within the gland as the organism develops from stages 1 to 4. The total protein content of the gland decreases as the DNase rises most sharply between stages 3 and 4. The curve on the right indicates that the greatest increase in specific activity of the enzyme, DNase, occurs between stages 3 and 4.

range of 0.10 to 1.32 μg of crystalline DNase I; the former value occurs in the mid-4th instar larva, 0.38 μg equivalents are present in the early prepupa, while an average 1.04 μg equivalents of antigen precipitate in the late prepupa. The measurements suggest that the changes in enzyme activity during glandular growth and development reflect changes in concentration of protein antigen rather than the more indirect action of interfering substances that lead to changes which alter enzyme activity.

DISCUSSION

IMMUNOLOGICAL ANALYSIS OF SALIVARY DNASE: The increase in total enzyme activity per gland is similar whether it is measured enzymatically or immunochemically; the magnitude of the increase by both assays is about 7-fold. The specific activity (precipitable protein/total protein) of the DNase in the gland determined immunochemically parallels the specific activity of the enzyme determined enzymologically during development. We conclude that the increase in DNase activity and precipitable antigen during development reflects changes in the concentration of the specific protein, deoxyribonuclease. The immunological cross-reactivity of dipteran and bovine DNase is perhaps surprising but suggests that the two enzymes may be similar in certain respects.

STATISTICAL SIGNIFICANCE OF DNASE ACTIVITY: One reason that analyses of closely related stages were not always significantly different from each other lies in the magnitude of the changes in the activities observed; these activities represent a developmental continuum and therefore include considerable overlap. A second factor which affects the significance of these particular comparisons is the unavoidable variability in the biological material. Nevertheless, we believe that the major conclusion to be drawn from the data is clear: the DNase activity changes appreciably, progressively, and significantly in the larval salivary gland during development.

DNASE AND SALIVARY GLAND DESTRUCTION: The increased DNase activity in the salivary gland at the end of larval life represents an increased enzyme activity that is restricted to a particular time in development, the beginning of metamorphosis. The salivary gland breaks down during metamorphosis while other tissues are undergoing extensive increases in size and in cell number. The destruction of this gland is presumably under hormonal control since glandular growth and breakdown have been shown to be

endocrine-dependent in a closely related species, *Drosophila virilis* (Bodenstein, 1943).

DNase may play an important role in changes within the gland for its activity is sufficient to account for the subsequent developmental events observed. The progressive decrease in protein content, pycnosis of salivary gland nuclei, and ultimate destruction of the entire organ seem to depend at least in part on DNase activity. This does not preclude the possibility that the DNase has additional and possibly entirely different functions.

Other enzymes may participate in glandular breakdown. Our own data (Laufer and Nakase, unpublished) indicate that at least one additional DNase with a more acid pH optimum exists in the gland. Protease and other hydrolases also exist in the gland (Laufer, 1963), and these could contribute to glandular destruction.

CHROMOSOMAL DIFFERENTIATION AND THE CYTOPLASM: Our observations of increased DNase activity show that biochemical changes occur in the protein composition of the salivary gland at critical times in development when chromosomal loci are observed to also undergo characteristic alterations (*cf.* Clever, 1961; Vogt-Köhne, 1961; Becker, 1962; Beermann, 1962; Kroeger, 1963; and Laufer, 1963). New chromosomal puffs appear at particular loci, while others regress. Particularly relevant are the studies by Vogt-Köhne and Kroeger since they are likewise concerned with *Chironomus thummi*. The puffed regions are thought to represent specific gene loci in phases of heightened activity. Puffs occur in specialized patterns in various tissues of the body, implying that different metabolic functions are associated with each region of the chromosome. Functional differences of puffed regions are also suggested by the differential incorporation of radioactive precursors into RNA at different puffed sites (*cf.* Pelling, 1959).

CHROMOSOMAL DIFFERENTIATION AND SPECIFIC PROTEINS: A close relationship between chromosomal activity and cytoplasmic product was suggested by the findings of Beermann (1961), who observed a correlation between the activity of one large chromosomal puff, a Balbiani ring, present only in a few cells of the gland, and the existence of cytoplasmic granules which also occur in the secretion of that gland.

Several classes of chromosomal puffs have been observed. These include loci that are continually active throughout most of the existence of the larval salivary gland. These are presumably associated with general metabolism, and "household" functions such as the production of the secretion. Many puffs presumed to be associated with general functions are turned off during the onset of pupation. Others appear during development, and therefore are presumed to be concerned with developmental processes. Of considerable significance in this regard were two sites that responded promptly when stimulated with the molting hormone, ecdysone (Clever, 1961). These loci are activated relatively rapidly following treatment with molting hormone. Other loci of developmental interest are those that undergo alteration at the time of molting and development. Some of these puffs are active during each larval molt, while others appear to be functional only at metamorphosis, and thus are only noted to change at the end of the 4th instar stage. The changes observed in DNase activity during metamorphosis occur at the same time and coincide with changes in puffing pattern. DNase activity should be associated with such stage-specific development-dependent puffs responsible for the active accumulation of the enzyme in the gland. Such puffs should also be dependent on hormonal regulation. Since the growth and destruction of the gland and the chromosomal puffing activity are dependent on hormonal interactions (*cf.* Bodenstein, 1943; Clever, 1961; Becker, 1962; and Laufer, 1963), hormones in all probability also control the DNase activity, albeit in some unknown way.

Lending further support to the idea that development is dependent on chromosomal activities are the findings of Laufer, Nakase, and Vanderberg, (1963 *b* and 1964). The use of actinomycin D revealed that specific chromosomal puffing, enzyme synthesis, and development all rely on DNA-dependent RNA synthesis. The most sensitive of these processes to inhibition was development. The increased sensitivity of larvae to actinomycin at metamorphosis was interpreted as indicating a dependence either on heightened nuclear RNA synthesis or on synthesis of new kinds of RNA. Others have also suggested that the puffs represent specific sites of messenger RNA synthesis (*cf.* Edstrom and Beermann, 1962; Sirlin, Jacob, and Kato, 1962; Clever and Beermann, 1963). According to this view, increased DNase activity results from the activation of one of the puffs that changes in activity only at the time of metamorphosis; subsequent develop-

mental changes in the gland result in its destruction.

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