PEPTIDASE INCREASE ACCOMPANYING GROWTH OF THE LARVAL SALIVARY GLAND OF DROSOPHILA MELANOGASTER*‡

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(Received for publication, December 10, 1948)

The study of enzyme synthesis during cellular growth is simplified by the use of a single type of cell whose growth proceeds under precisely defined conditions. For such experiments the larval salivary gland of *Drosophila melanogaster* is good material. The cells are of one kind, they increase in size without any division, and finally they break down during pupal metamorphosis. Hence the processes attendant on cell growth can be studied without the complications of mitosis, and the contrasting changes at cell breakdown can be followed. Moreover, *Drosophila melanogaster* is an organism in which the genetic and nutritional factors of growth can be varied with precision: the fly is a classic object of genetics, and it can be grown on a chemically defined medium (Schultz, St. Lawrence, and Newmeyer, 1946).

Following the exploratory work presented in the preceding paper (Patterson, Dackerman, and Schultz, 1949), the measurement of alanylglycine (AG)-peptidase activity could be used as an index of the content of AG-peptidase during growth of the salivary gland. Cellular growth, that is, the increase in size and/or number of constituent metabolic units, is largely a function of protein synthesis. The peptidases occupy a position of special interest in these processes. Not only may these enzymes be synthesized during the growth of the cell, but their function, even as hydrolytic agents, may be connected with the growth process.

This paper presents evidence for an increase in AG-peptidase content accompanying the growth of the gland, contrasted with a decline in measurable activity at the onset of histolysis. The increase in enzyme parallels the increase in total nitrogen content of the developing gland. A similar relationship between enzyme content and total nitrogen is evident in glands of different sizes, but at the same developmental stage. These results, when considered together with the data of earlier workers, particularly Linderstrøm-Lang and Holter and their group (see Discussion), lead to the conclusion that peptidase

* Presented before the American Society of Biological Chemists at Atlantic City, March, 1948 (*Fed. Proc.*, 1948, **7**, 177).

‡ This work was assisted by a grant from the Pardee Foundation.

increase is a concomitant of cellular growth and suggest the possibility that these enzymes form an integral part of the synthetic system of the cell.

BIOLOGICAL MATERIAL

Characteristics of Salivary Glands

In spite of interest in the cytochemistry of the salivary glands aroused by the giant chromosomes of their nuclei, little is known of their physiology. Their development has, however, been studied both descriptively (Ross, 1939) and experimentally (Bodenstein, 1943). The larval salivary glands of *Drosophila* are paired structures joining into a common duct that opens into the pharynx of the larva. Full grown they are long and thin (Fig. 1), 1.0 to 1.7 mm. long by 0.16 to 0.25 mm. wide. Their average dry weight is about 5 μg., their average volume 0.02 μl. Each gland contains about 128 cells arranged about a central space, the duct. During larval life there is a thousandfold growth of the cells. With the approach of metamorphosis of the larva, the salivary glands begin to histolyze, finally breaking down completely except for the ring of imaginal cells destined to form the gland in the adult fly. These cells are adjacent to the duct and are easily removed when the ducts are cut off. The rest of the cells of the gland are all of one type. During development the cells at the tip are the first to attain a maximum size and degree of differentiation which is gradually approached by cells farther up the gland. The material used in the present work was limited to a late period of growth when the glands were large enough to permit easy removal of the adherent fat body. During this time most of the cells were in the final stages of differentiation.

The secretory function of the salivary glands of *Drosophila* is not well understood. Various staining techniques indicate an accumulation of secretory product at the end of the third instar. The secretion is trapped in the lumen of the gland at that time, possibly being given off in a mass during the subsequent formation of the puparium. After this time secretory globules are seen in the cells of the gland almost until definite histolysis sets in. Kodani (1948) has found that the secretion contains a protein material (10.5 per cent N) and has made a chromatographic determination of its amino acids.

Genetic Stocks

The material for these experiments is the same as that of the previous paper (Patterson, Dackerman and Schultz, 1949) but is described here more fully. Four stocks of *Drosophila melanogaster* were used. The first, a wild stock, + Tuscaloosa (Tusc), was only slightly inbred and was replaced in the experiments when on inbreeding it was discovered to contain a number of recessive mutants. The standard stock thereafter was a completely homozygous wild-type stock, + Oregon R (Ore R) (Bridges and Brehme, 1944); since its intensive use in this laboratory, it has been inbred for twenty generations by brother-sister matings. In addition to these two wild stocks, use was made of a sex-linked giant mutant (genetic symbol, gt; locus I, 0.01) in which about 20 per cent of the individuals have a prolonged larval life, pupating 2 to 3 days late as giant individuals (Bridges and Gabritschevsky, 1928). Two stocks of this giant mutant served as experimental material. The first (gt Ore R), made up to

contain Ore R autosomes, proved to be heterozygous for a second chromosome inversion (Cy 2R), and showed a variability which was too great for the later more precise experiments. The gt w^a Ore R strain was obtained from a series of crosses with stocks containing crossover eliminators marked by mutants. This resulted in the

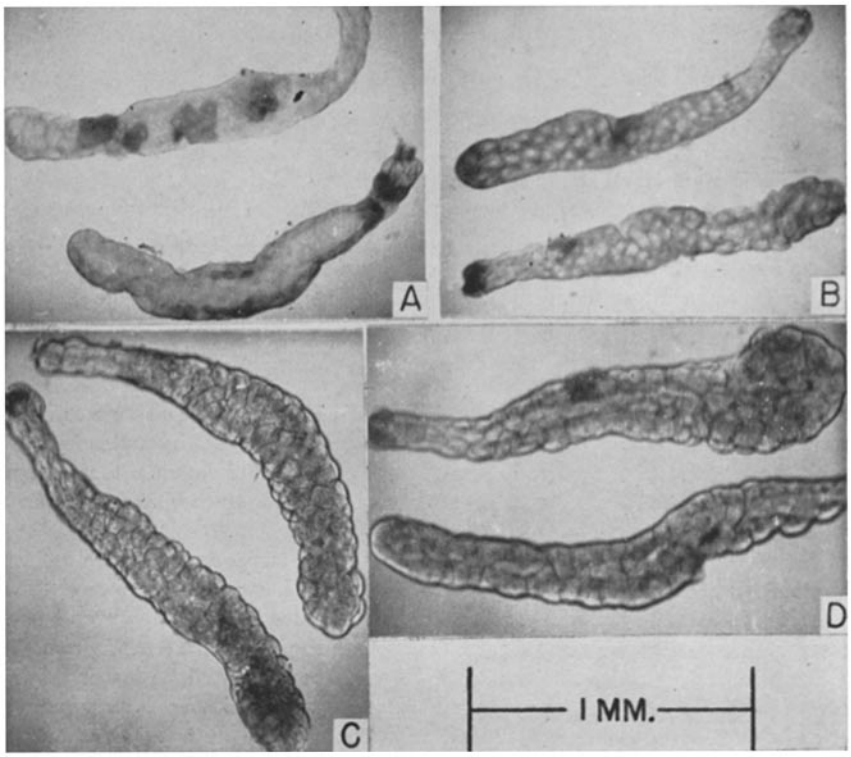

FIG. 1. Photomicrographs of larval salivary glands from prepupae of *Drosophila melanogaster*. A, pair of glands from an Ore R pupa 1 hour beyond stage ev. The AG-peptidase content of these glands is plotted in Fig. 2 at the points O_1 (gland at bottom) and O_2 (gland at top). Note the patches of dark cells in the upper gland indicating disintegration. B, C, D, pairs of glands from 5 hour prepupae of the gt w^a Ore R stock. Note the differences in size. B, from male non-giant prepupa, 120 hours after egg laying. C, from male giant, 185 hours after egg laying. D, from female giant, 168 hours after egg laying. × 38.

production of a stock homozygous for the giant mutant and the sex-linked eye color apricot. Except for that small section of the X chromosome, the stock is isogenic with the inbred Ore R.

It is to be noted here that the difference between giant and non-giant flies in this stock is not genetic (the stock is homozygous, giants and non-giants breed alike) nor is it, as Bridges and Gabritschevsky supposed, nutritional. It must rather be con-

sidered as a variability in embryonic determination, leading to a delay in the time of release of the pupation hormone (Morgan, Bridges, and Schultz, 1936). The stock thus has the value of supplying individuals of identical genetic constitution, raised under identical environmental conditions, but still of different sizes at comparable physiological stages.

EXPERIMENTAL PROCEDURE

Culture of Larvae and Pupae

In a typical experiment, eggs were collected from a mass culture of male and female flies, all these the progeny of a single pair. Since it was necessary to be fairly precise in the timing of the larval and the pupal stages in certain of the experiments, eggs were collected from a 3 to 4 hour egg-laying period. In the case of the giants where special precautions were desirable, larvae were collected at hourly intervals as they hatched from the eggs which had been deposited on a yeasted molasses agar medium. The eggs or larvae were transferred to the normal medium used for raising the flies, the standard corn meal molasses agar mixture fortified with brewer's yeast and seeded with fresh yeast. Fresh yeast was added after 2 days and for the giants again on the 5th day (25°C.).

As the inbred stocks are now constituted and with standard culture conditions ensuring maximal feeding, developmental rates show little variability and indeed the results from generation to generation check quite closely. Formation of the prepupa occurred at about 120 hours (at 25°C.) after egg laying in both the Ore R and the gt w^a Ore R non-giant larvae.

The stages of development (*cf.* Robertson, 1936) of the larvae and pupae from which the glands were dissected are defined as follows. "Late Larvae" (LL) are those third instar larvae that have crawled up the side of the culture bottle and are no longer feeding. Later prepupal stages are defined by the time elapsed at 25°C. from the moment (stage 0) at which the larva, with the pupal horns everted, no longer moves. Timing is necessary for the determination of the stages up to 3 hours after puparium formation. From 4 to 9 hours, identification is facilitated by a large gas bubble whose size and position in the prepupa is characteristic for each hourly stage. Later than 9 hours, one or another of the defined earlier stages was used for timing until pupation proper, with the eversion of the imaginal discs, occurred at about 12 hours. Glands from pupae in which the imaginal heads have just everted are at the "everted stage" (ev). The larval glands at this stage are at various degrees of disintegration, although still grossly whole in appearance (see Fig. 1 A).

Dissection

The dissection procedure was exactly as described in the preceding paper. All experiments were carried out in an air-conditioned, dust-proof room, and dissection was performed on a cold stage of a binocular microscope.

Measurements of Salivary Glands

1. Volume.—Since it was necessary to measure the volume of the glands, the procedure followed when single glands were used differed from that described for extracts

in that a pair of glands was photographed while in the drop of Ringer's solution. The whole operation of photography took about 1 minute, thus the glands did not warm up appreciably. The photographs served not only for volume calculations, but also as records of the condition of the glands.

An Argus camera carrying 35 mm. film was fitted to one ocular of the binocular eyepiece of a compound microscope (16 mm. objective, 2 × ocular), the other ocular being used for focusing. The area of the enlarged image of the negative was determined by using a planimeter and the greatest width was measured. The volumes were calculated assuming an ellipsoid shape for the glands ($V = 2/3\ Ad$, where V is the volume, A the area, and d the diameter).

2. Nitrogen.—Nitrogen determinations in the range of 1 μg. are necessary for objects the size of the salivary gland. This amount of nitrogen may now be determined very accurately by the method of Brüel, Holter, Linderstrøm-Lang, and Rozits (1947) but this method was not available at the time these experiments were carried out. Our procedure was based on the Levy-Palmer (1940 *a*) decimicro adaptation of the Theorell (1928) sodium hypobromite method. It allows a direct determination of ammonia in the acid digest in the original tube and in this respect is simpler than the Carlsberg method which involves a transfer from tube to tube and a distillation of ammonia.

The decimicro method of Levy and Palmer was first used to determine total nitrogen in ten salivary glands. They were found to contain 2 to 8 μg. nitrogen. It was, therefore, necessary to refine the method and reduce the quantities by a factor of ten in order to determine nitrogen in one gland. Levy constriction type micro pipettes were used throughout and the final titration carried out with a type II Linderstrøm-Lang burette (Linderstrøm-Lang and Holter, 1940).

Details of the procedure used for the determination of total nitrogen in individual salivary glands are as follows: Each gland with the fat removed was transferred on a glass needle from Ringer's solution through a drop of double distilled water onto a small (2 × 2 mm.) coverslip. This was picked up with watchmaker's forceps and dropped into the digestion tube (Kimble glass precipitin tubes, 6 mm. diameter, 50 mm. long) containing 10 μl. water. Ten μl. of 1:1 H_2SO_4 was then added and the digestion carried out with the lower 5 mm. of the tubes resting in depressions in a heated and insulated aluminum block. The open ends of the tubes projected into a horizontal glass cylinder (1 inch diameter) through a slot (½ × 12 inches) cut into its lower side and thus were covered. Superoxol and potassium persulfate were used as additional oxidizing agents.

The total digestion time was about 8 hours at 210°C. Neutralization, addition of sodium hypobromite, acidification, and addition of solid KI were carried out in the original tube and at carefully timed intervals. The iodine released was immediately titrated with N/20 sodium thiosulfate. All operations were carried out in a scrupulously clean, air-conditioned, dust-proof room.

At the present stage of the work, nitrogen can be determined in the range 0.9 to 2.5 μg. with an error of ±0.01 μg. Below 0.9 μg., while the routine error remains the same, the percentage error obviously increases. A few "outsiders" (see Brüel, Holter, Linderstrøm-Lang, and Rozits, 1947) also begin to appear. Nevertheless, the hypobromite method has been used in these experiments for determinations down to 0.4

μg. nitrogen. As seen from the figures given in Table I, the percentage variability in the total nitrogen values is about the same as that in the enzyme determinations and less than that in the volume determinations.

3. Peptidase.—Determination of the peptidase content of individual glands was carried out by the titrimetric micro methods of Linderstrøm-Lang and Holter (1940).

TABLE I

Peptidase Content, Total Nitrogen, and Volume of Salivary Glands in Various Stocks of Drosophila melanogaster

Stock	Experiment No.	Sex	Stage	Age*	Peptidase‡				Nitrogen				Volume				Peptidase / Nitrogen
					M§	σ_M	Var.	N	M	σ_M	Var.	N	M	σ_M	Var.	N	
				hrs.	*μl. HCl*		%		*μg.*		%		*μl. × 10^3*		%		
Tusc	1.08–1.10	♀	LL	120	3.0	0.3	31	11									
Ore R	2.01–2.13		LL	120	3.2	0.3	25	9					32	3	19	5	
			4–5	120	5.3	0.4	22	8	0.53‖				33	3	21	7	9.9
			6–7	120	6.3	0.5	22	7					40	3	22	7	
gt w^a	4.02	♀	5	120	4.0	0.3	19	8	0.67	0.03	13	9	32	1	18	20	6.0 ± 0.5
Ore R	4.03		5	120	4.7	0.2	15	8	0.67	0.03	12	8	33	2	24	20	7.1 ± 0.5
	4.03		5	167	9.2	0.3	10	11	1.00	0.03	9	12	60	2	17	21	9.1 ± 0.4
	4.02		5	187	8.4	0.2	5	5	1.11	0.09	16	4	64	2	13	17	7.5 ± 0.6
	4.03		5	187	8.7	0.6	16	5	1.04	0.02	5	5	58	3	15	9	8.4 ± 0.6
Tusc	1.08–1.10	♂	LL	120	2.5	0.1	20	13									
Ore R	2.01–2.13		LL	120	2.6	0.3	30	5					30	2	16	5	
			4–5	120	4.5	0.4	19	5	0.47‖				25	3	17	3	9.5
			6–7	120	4.6	0.3	16	8					31	2	15	8	
gt w^a	4.02	♂	5	120	2.6	0.2	16	7	0.54	0.06	13	7	24	1	15	20	4.9 ± 0.4
Ore R	4.03		5	120	3.0	0.2	14	8	0.48	0.03	16	9	26	1	15	20	6.1 ± 0.4
	4.02		5	167	6.3	0.3	10	4	0.81	0.05	14	5	50	3	18	8	7.8 ± 0.6
	4.03		5	167	6.2	0.4	15	5	0.75	0.03	12	7	47	2	17	14	8.3 ± 0.7

* Hours after egg laying.

‡ Hydrolysis of 0.18 M alanylglycine by one gland in 4 hours at 40°C., pH 7.65, measured as the titration value; *i.e.*, μl. N/20 HCl in 97 per cent alcohol. Titration values are read to the second decimal place; accuracy of blanks is ±0.08 μl. In Experiment 4.03 the time of hydrolysis was reduced to 3 hours and the readings corrected to the standard 4 hour period.

§ Mean (M); standard error of the mean (σ_M); coefficient of variability (Var.), σ/M 100; No. (N) of determinations.

‖ From a single determination of total nitrogen in ten glands.

Details of the technique as applied to extracts of the glands were given in the preceding paper. The same procedure was carried out with the single glands except that instead of using an aliquot of gland extract as source of enzyme, here a gland was added to a 7 μl. aliquot of glycerine buffer (M/60 phosphate, 30 per cent glycerine) previously pipetted into the reaction vessel. In contrast to the experiments with extracts, the tissue was present during the enzymatic reaction.

In the experiments with the Ore R and Tusc stocks the glands were introduced into the buffer drop by means of a glass needle. Occasionally the glands stuck to the needle necessitating a return to the rinsing buffer drop to loosen them. This was found to cause a slight loss of enzyme. Therefore, in all the work with the gt w^a Ore R stock, the glands were transferred from the buffer drop to a small (about 2 × 2 mm. square) clean coverslip and the coverslip picked up with watchmaker's forceps and dropped into the tube (Holter and Doyle, 1938). Check experiments showed that this change in technique made no difference in the activities measured.

Immediately after a pair of glands was placed in the buffer drops, the two tubes were rapidly frozen (in dry ice–acetone) and thawed seven times. An additional tube containing a drop of buffer (reagent blank) was frozen and thawed with each alternate pair of glands. After an hour's extraction at room temperature the tubes were kept frozen over dry ice, until 5 minutes before substrate was added. (A time of extraction longer than 1 hour gave no increase in enzyme activity.) The tubes rested in holes in a disc of lucite cut to fit the small Dewar flask containing dry ice. No activity was lost after weeks of storage.

The process of freezing and thawing ensured sterility of the preparations, but did not produce cytolysis of the cells of the glands. In an attempt to ensure cytolysis, glands were placed in M/30 phosphate buffer, frozen and thawed, then extracted an hour before addition of 60 per cent glycerine (giving final concentration as above). Not only were the glands not cytolyzed, but the splitting of alanylglycine obtained was lower than that of similar glands extracted in glycerine buffer. Without the stabilizing effect of glycerine, the enzyme rapidly becomes less active, as is typical of this peptidase (Linderstrøm-Lang, 1929). Since standard results were obtained using glycerine buffer, the use of this procedure, which did not include cytolysis, seemed justified.

dl-Alanylglycine (0.18 M, pH 7.60 ± 0.05 at 40°C.) served as substrate for the peptidase determinations. In the previous work with extracts, the splitting of this peptidase was found to give a good index of peptidase content of the glands. Blanks were run as in the experiments with extracts except that with the single glands one gland of a pair was used for the "determination" and the other for the "enzyme blank." The enzyme blank values were found to be low and fairly constant, averaging 0.20 μ1. N/20 HCl per gland (Ore R and Tusc) comparable to the values found in the gland extracts. In the final series of experiments, enzyme blanks were omitted, the second gland of a pair being used for a total nitrogen determination.

The reaction time in all experiments except those mentioned below was 4 hours at 40.0°C. Under the experimental conditions used (Patterson, Dackerman, and Schultz, 1949) degree of hydrolysis through a titration value of 6 μl. N/20 HCl was found to be proportional to concentration of extract and time of reaction. In the first experiment with the gt w^a Ore R stock, the splitting was found to exceed this value and the reaction time was subsequently cut to 3 hours.

Determinations of endopeptidase activity were carried out on single glands as described in the preceding paper for extracts. α-*N*-Benzoyl-*l*-argininеamide served as substrate and the reactions were carried out in citrate buffer at pH 5 with 0.01 M cysteine present. The reaction time was 20 hours at 40°C.

RESULTS

Three sets of experiments carried out under different biological conditions give information concerning the relation between peptidase and mass of the glands. The first set (measurements during the prepupal instar after food intake has ceased) deals with changes during the late stages of cell growth and differentiation and during the period of cell breakdown. The second set (measurements covering the late larval life of the giants) gives similar data for that period in which the cells are closer to the grand period of growth and in which the physiological state of the gland may still be conditioned by food intake. In the third group, genetically identical individuals of different sizes in the giant stock allow a comparison of glands at the same stage of development, but differing in mass. Considered individually, each set of experiments does not allow an unequivocal conclusion; collectively, however, they present a consistent picture of increase of peptidase during growth.

Peptidase Content and Total Nitrogen in the Larval Salivary Gland of the Oregon R Stock during the Prepupal Period

Fig. 2 presents measurements of the AG-peptidase content of salivary glands over a 14 hour period including the last stages of growth and the first stages of histolysis. Glands were taken from Ore R larvae just before puparium formation (stage L), and thereafter from prepupae at hourly intervals until eversion of the imaginal discs (pupation proper).

Curves of enzyme content in both males and females show a steady rise in the amount of hydrolysis of AG until the 6 to 7 hour period, the difference between the peak value and the initial measurement being statistically significant. Subsequently the peptidase content declines, with a considerable increase in variability. Both of these phenomena appear to result from the onset of histolysis, which is evident in the opaque patches on the glands after the 6 hour stage.

The correlation between histolysis and a lowered AG-peptidase content is illustrated by a comparison of the individual values given at points O_1 and O_2, and $\times_1$ and $\times_2$. The points O_1 and O_2 correspond to the peptidase contents of the two glands of a pair given in the photomicrograph in Fig. 1 A. The glands were dissected from a female pupa 1 hour after eversion. The gland at the bottom of Fig. 1 A shows little disintegration and it had a very high peptidase content, O_1. Several opaque spots are clearly visible in the gland at the top and its peptidase content, O_2, was significantly lower. In contrast, the greatest difference in peptidase content between glands of a pair in the stages up to 6 hours, *i.e.* before histolysis begins, was 5 per cent. The values $\times_1$ and $\times_2$ represent the highest and lowest peptidase contents found in glands from 11 hour male prepupae. $\times_1$ is the value from a gland showing no visible sign of disintegration; $\times_2$, the value from a gland of similar size with

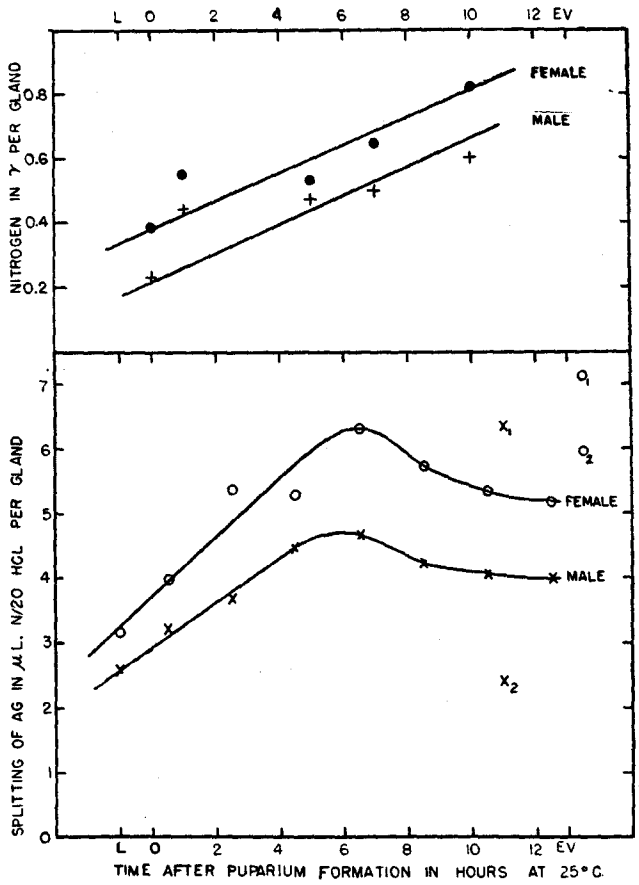

FIG. 2. AG-peptidase and nitrogen during the period of growth and breakdown of the larval salivary glands in the Ore R prepupa. Peptidase is plotted as the titration value; *i.e.*, the hydrolysis of AG in microliters of N/20 HCl in 4 hours at 40°C. The means of 5 to 12 determinations of the peptidase content of individual glands, grouped in intervals of 2 hours, are plotted in the lower graph. X_1 and X_2 represent the highest and the lowest of the individual male values at 11 hours; O_1 and O_2, determinations made on two glands of a pair from an everted female pupa (see Fig. 1 A). Nitrogen determinations on ten glands are plotted in the upper figure.

many of the opaque patches that are characteristic of breakdown. In X_1 as in O_1 the enzyme content had increased over the values at earlier stages: disintegration of the "mature" cells had not yet begun, while the cells that were still growing continued to add to the enzyme content of the whole gland. In X_2, where disintegration was well on its way, activity was decreased: the cells which had broken down subtract from the total possible enzyme content. It

seems likely, therefore, that per cell, the peptidase content increases until breakdown begins. Thus, the activity of the peptidase is not maintained at the time when the proteins of the salivary gland presumably become available for hydrolysis.

The fall in exopeptidase activity with disintegration of the gland is paralleled by a rise in endopeptidase activity (the ability to split benzoyl-*l*-arginineamide (BAA) at pH 5 with cysteine present). These experiments were carried out with the Tusc stock. When individual glands were used, from either late larvae or pupae in which the glands were still in the synthetic phase, no hydrolysis of BAA was found even with a 20 hour reaction time. But when glands from everted pupae were tested, that is glands that showed opaque spots indicating disintegration, a measurable but small hydrolysis of BAA occurred, averaging 0.30 μl. N/20 HCl per gland. This corresponds to the value found in extracts of glands (Patterson, Dackerman, and Schultz, 1949). Since the splitting with the single glands was low, two glands were used in many of the experiments and the time of extraction was cut to zero in order to keep the autolytic value (enzyme blank) small. Even so, this ranged from 0.06 to 0.84 μl. N/20 HCl when the hydrolytic value was 0.48 to 1.08 μl. N/20 HCl. The variability in degree of hydrolysis corresponded to the variability in the degree of disintegration shown by the glands.

The interpretation of the changes with time in terms of actual growth requires the use of some index of total mass. Reduced weight, as used by Linderstrøm-Lang and Holter (1940), and the Cartesian diver balance of Zeuthen (1947) provide methods of weighing objects of this size but could not be used at the time of these experiments. Volume measurements provided an index of sorts, but with obvious weaknesses. Actually as may be seen in Table I (Experiments 2.01 to 2.13), the volumes found in this period were highly variable and showed no consistent trend. Since there are visible changes in the size of the lumen of the gland (secretion ?), it was concluded that volume is not a satisfactory index of cell mass during the prepupal period. The measurement of total nitrogen appeared to be more useful in the case of the salivary gland in which no obvious large bodies of nitrogenous storage material are present. The nitrogen measurement has a major advantage from a theoretical point of view. Since the peptidases are probably largely protein, the total nitrogen gives a measure of all possible precursor nitrogen available for the synthesis of the enzyme.

Nitrogen values determined on groups of ten glands of the respective pupal ages are shown in the upper graph of Fig. 2. The points fall roughly on a line, with the exception of the 1 hour stage, at which a high value is found, possibly connected with a retention of the protein-containing secretion. During the prepupal period, the total nitrogen of the glands increases almost 100 per cent up to the time of breakdown. During the period of disintegration, it appears

that there is no appreciable release of nitrogen arising from the histolysis of protein and the diffusion of free amino acids from the gland. The enzyme blanks show very little change suggesting only a small increase in the concentration of free amino acids. Apparently the process of protein breakdown has not proceeded to completion at this stage.

It is evident that the peptidase and nitrogen content of the salivary glands in the prepupal period increase together, up to the onset of histolysis. After that time they diverge, the nitrogen continuing to increase while the peptidase declines. The interpretation offered is that the peptidase activity persists only in those cells in which histolysis has not yet occurred, whereas the nitrogen increase is due to continued growth in the "younger" cells of the gland.

Peptidase, Volume, and Nitrogen Content of the Giant Salivary Gland during Larval Growth

The next step was to determine whether the increase of peptidase with total nitrogen is confined to the prepupal period, or is a general accompaniment of cellular growth of the gland. The grand period of growth occurs in the minute glands of the early larva which are difficult to deal with experimentally. But growth can be studied in the giant stock where the extra larval period provides individuals with large glands in which peptidase and nitrogen measurements can easily be made.

The survey of peptidase and nitrogen content made in the following experiments covers the latter third of the larval development of the giants produced in this mutant stock (gt Ore R). Larvae and/or pupae (stages 1, 2, 5, 8), in approximately equal number for each sex and stage of development, were collected at 10 and 20 hour intervals between 110 and 210 hours after egg laying. There are ordinarily two peaks of puparium formation in the larvae collected at one time from a given batch of eggs. One peak (non-giants) occurs at the normal time of puparium formation, about 120 hours (25°C.) after egg laying. The other peak (giants) occurs after a delay of 2 or 3 days. In the intervening time, the giant larvae can be collected. In this particular strain (see Genetic stocks) there were a few laggard non-giant pupae and there was a greater spread in the time of pupation of the giants than in the gt w^a Ore R stock used in subsequent experiments.

Mean values of peptidase contents of glands from three experiments are plotted in Fig. 3 A. Because of the variability in the stock, the data showed too great a scatter to permit a detailed study of the relations in the separate prepupal stages. These values were therefore grouped and used as a gross criterion of the final stage of larval development for a given time after egg laying. The data from the two sexes were also combined. It is clear that during the extra larval growth period of the giant, the peptidase content of the salivary gland rises with time. Thus a peptidase increase accompanying

growth is also characteristic of this larval period and is not merely a physiological peculiarity of the prepupal period.

When the individual values were inspected the data for the different prepupal stages allowed a rough comparison of the time relations of peptidase

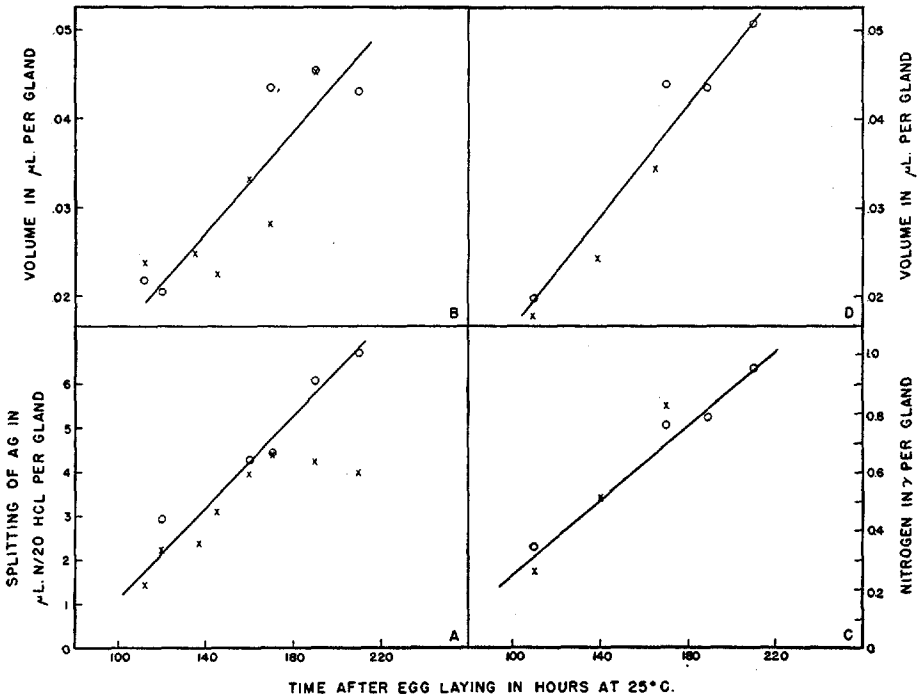

FIG. 3. Peptidase, nitrogen, and volume during larval growth of the salivary glands of the gt Ore R stock. Means of peptidase values (graph A) from larvae (×) and prepupae (○) are plotted separately but values from males and females and from the different prepupal stages are grouped. Each point represents the mean of 4 to 10 values included in ±5 hours from the time after egg laying plotted. The mean values of the volumes from these same glands are plotted above in graph B. Graph C gives mean nitrogen values from a similar experiment. The volumes of the glands used in the nitrogen determinations are plotted in graph D.

change in the giant and non-giant individuals. Apparently, both in the giant and non-giant glands, the peptidase content is maximal at the 5 hour prepupal stage, thus agreeing with the results obtained on the Oregon R stock (Fig. 2). This result is of importance since the possibility existed that histolysis might begin earlier in the giants with their prolonged larval period.

After 180 hours, there are a few abnormally low larval values coming from individuals which eventually die without pupating. These glands may already

be undergoing some disintegrative changes of the type shown to occur in the normal gland just before the breakdown of metamorphosis. The low peptidase values would be consistent with such an onset of disintegration (see previous section).

The volumes of the glands from these experiments are given in Fig. 3 B. These measurements, made as described above, are approximate but sufficient to show that the peptidase increase is accompanied by an increase in volume. The volumes of the abnormal 190 hour larvae are high even though the peptidase is low, again suggesting that disintegration is lowering the peptidase value as in the pupal glands.

In order to correlate the peptidase and volume data with the total nitrogen of the glands, a similar experiment was carried out, in which nitrogen determinations were made on pairs of glands whose volumes were also measured. The results appear in Figs. 3 C and 3 D respectively. Both nitrogen and volume have increased as did the peptidase content and volume of the glands in the comparable experiment (Figs. 3 A and 3 B). The volume measurements are more variable than are those of total nitrogen; the latter are obviously a more accurate means of estimating the total protoplasmic mass. The volume measurements do show that variations in water content during this larval period must be on the whole minor to allow the general agreement with the nitrogen values.

It may be concluded, therefore, that during the larval growth of the gland in the giants, the increase in AG-peptidase is correlated with an increase in total nitrogen. But this increase, both in nitrogen and peptidase, occurs at a slower rate in the giant larva than in the Ore R prepupa previously examined. The time required for the peptidase content in the prepupa to double is only 6 hours whereas it is 50 hours in the larval giant gland. This difference poses a problem for future work. For the present, the important point is that in the two systems, where the absolute rates of increase are so different, the peptidase-nitrogen relations are similar.

Peptidase Content in Relation to Total Nitrogen in Glands from Giant and Non-Giant Prepupae

The measurements at the different stages of development described in the experiments of the two previous sections do not allow a discrimination between changes due to growth proper, and those due to differences in phase of the secretory cycle of the glands. In order to make such a discrimination, it seemed advisable to compare the peptidase-nitrogen relation in glands of different sizes but at the same physiological stage. Here differences in enzyme content would be directly related to cell size.

For this study the 5 hour prepupal stage of the giant and non-giant individuals was chosen. At this stage the peptidase content is at a maximum and the

fat body most easily dissected away from the gland. Males and females of the giants and non-giants provide a range of sizes sufficient to establish a relation between peptidase and nitrogen. As already pointed out, the existing data indicate that the change in peptidase content during the prepupal period is similar in these four types, allowing a valid comparison between them at the 5 hour stage.

The experiments (see Table I) were carried out on the rigorously inbred giant apricot Oregon R (gt w^a Ore R) stock. 5 hour non-giant prepupae were taken at 120 ± 2 hours after egg laying. Under optimum conditions the majority of the giant individuals in this stock have reached the 5 hour prepupal stage at 167 ± 4 hours. There are a few stragglers up to 187 hours. In Experiment 4.02 the female giants could not be collected at the proper time and the few later pupae were used. To allow comparison of individuals from the two different groups, 5 hour prepupae were collected at 167 ± 3 and 187 ± 3 hours (Experiment 4.03).

The experiments were further refined in that nitrogen determinations were made on one member of a pair of glands, peptidase determinations on the other. Volumes were calculated for all the glands used. Previous experiments had shown that the differences in peptidase content between members of a pair were not more than 5 per cent (Ore R), differences in total nitrogen values not more than 10 per cent (gt w^a Ore R), and differences in volume not more than 5 per cent (Ore R). The mean values obtained in two separate experiments of this kind (Experiments 4.02 and 4.03) are presented in Table I. The individual values for Experiment 4.03 are plotted in Fig. 4, the ordinates being the amount of hydrolysis of alanylglycine found in one member of a pair of glands, the abscissae being the total nitrogen found in the other member of the pair.

It is evident that in both experiments, the higher the total nitrogen, the higher the peptidase content. However, the ratio between the two quantities is not constant: at the higher nitrogen values of the giant groups, the average peptidase-nitrogen ratio is over 8, as compared with the lower ratio around 7, of the non-giant groups. This difference in ratio may indicate a situation in which part of the nitrogen increases directly with the AG-peptidase, while the remainder is unrelated to this enzyme. Further data defining more closely the nature of the curve of enzyme increase during development are clearly needed before such a discussion could be profitable. The emphasis at present is placed upon the fact that in glands of different sizes, at the same physiological stage, the AG-peptidase content of the gland goes along with the increase in mass as measured by total nitrogen.

Thus from the three different sets of experiments, the same conclusion follows: the higher the total nitrogen, the more peptidase, until histolysis begins. It seems a reasonable conclusion from the experiments of the present section that

this increase is concomitant with the "true" growth of the gland and is not merely a reflection of changes in the secretory cycle. It is difficult to conceive that the parallelism between peptidase content and total nitrogen would otherwise be maintained under the three sets of conditions. Only if secretion were continuous and formed a constant proportion of the total nitrogen could such a result be obtained, and in that case the secretion would be essentially a con-

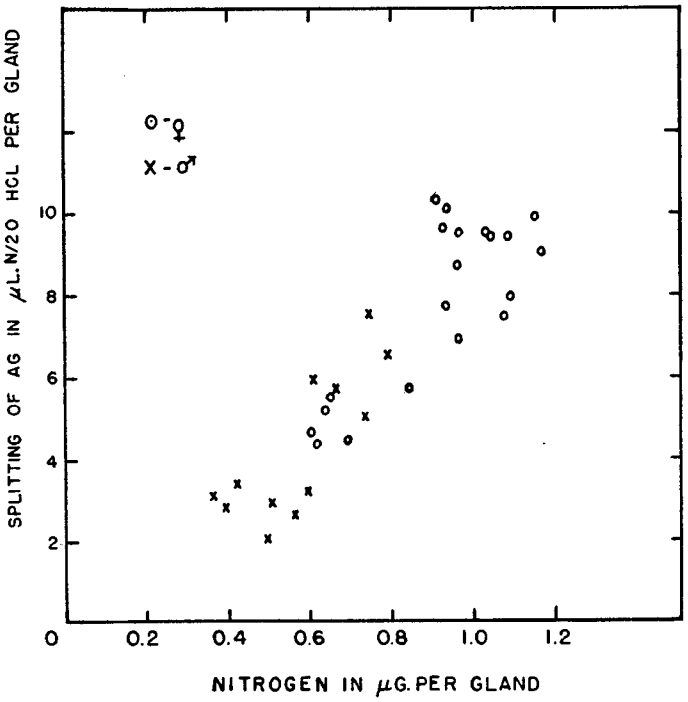

FIG. 4. The relation between peptidase and nitrogen in 5 hour prepupal glands from gt w^a Ore R males (X) and females (O). Peptidase determinations were carried out on one gland of a pair, nitrogen determinations on the other.

stant component of the cell system. Actually what evidence there is, as already stated, points to a peak of secretion just before puparium formation. Yet the above data give no indication of any major effect of this process on either the peptidase or the nitrogen.

Relation between Peptidase Content and Genetic Factors

It is obvious that enzymes with the high degree of specificity shown by the peptidases are suitable material by means of which a study of the relation between genes and enzymes can be approached. The present study, while tangential to this objective, gives some information of a preliminary character.

Knowledge of the enzyme spectrum of the salivary gland should make it possible to screen the different mutants in *Drosophila* for differences in content of specific enzymes. Since this paper is concerned with only one enzyme, and mainly with only four genotypes (males and females of the giant and Ore R stocks) these data could only give evidence of striking quantitative differences.

The information is most complete concerning these relations between male and female. The peptidase content of salivary glands of males is consistently lower than that of the females in all the stocks studied. The data of Fig. 4, already discussed, show that this low value is correlated with a correspondingly low nitrogen content. Thus it appears that the difference in peptidase content between male and female is the result of a difference in mass, corresponding to the well known fact (Dobzhansky 1929) that the cell size of the male is smaller than that of the female.

The interdependence of peptidase content, total nitrogen, and volume measurements as between males and females is strikingly borne out by a comparison of the sets of values from the different experiments. When the values for males are plotted against the corresponding ones for females (Fig. 5) all these points for peptidase, volume, and nitrogen fall on the same straight line: the ratio (1:1.35) between males and females is constant for all points. The sexual difference in the peptidase content has the same ratio as the difference in mass.

The graph discussed contains one additional set of points which necessitates a slight digression. The values obtained in experiments using extracts of the Ore R and Tusc stocks (Patterson, Dackerman, and Schultz, 1949) fit on the same part of the line, *i.e.* have the same absolute values, as those obtained from experiments in which the whole gland was present, showing the completeness of extraction of the enzyme.

The dependence of male-female relationships on differences in mass reinforces the main thesis of this paper. It does not give indications of genetic influences in a specific way on peptidase content. A difference deserving some note was found between two strains of the first stock of the giant mutant used (gt Ore R), in which only the autosomes were of Ore R ancestry. One of these strains gave high peptidase values, the other lower values and showed a much higher variability. It was found that the variable stock actually was heterozygous for an inverted section of chromosome II, introduced into the stock by an undetected rare crossover during its preparation. The use of the stock was thereupon discontinued; but the difference due to change of chromosome composition is pertinent.

A more definite difference, which holds promise for future work, is found in the comparison of non-giants from the gt w^a Ore R stock with individuals from the Ore R stock having a similar nitrogen content. The Ore R peptidase values are high relative to the non-giants although they agree fairly well with earlier

values on another wild type stock, Tusc. The AG-peptidase-nitrogen ratio is, however, the significant quantity (see Table I), being lower in both males and females of the non-giants (120 hours) of Experiments 4.02 and 4.03 than it is the Ore R individuals of a similar age and nitrogen content in Experiments 2.01 to 2.13. The meaning of these differences is a problem to be analyzed in

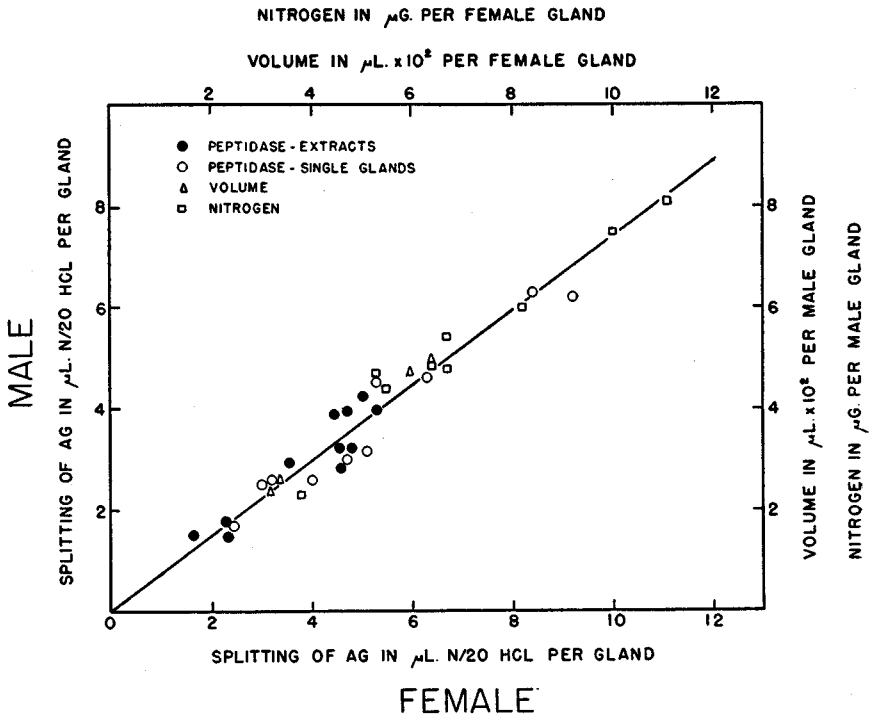

FIG. 5. Male:female relationship in peptidase, nitrogen, and volume determinations made on salivary glands. The line represents ♀ = 1.35 ♂. The points plotted are means of determinations made on glands of various stages from the Tusc, Ore R, and gt w^a Ore R stocks. The peptidase values for extracts of glands from the Tusc and Ore R stocks were taken from data of Patterson, Dackerman, and Schultz (1949).

detail, on separate isogenic gt and w^a stocks, upon which measurements of the course of peptidase increase must be carried out. Thus, in addition to the general relation to growth, the closer study of this material should hold interest for chemical genetics.

DISCUSSION

Histochemical estimations of peptidase content have the limitations noted by their originators (see Linderstrøm-Lang and Holter, 1934). An assay of

the total amount of enzyme content is obtained by measuring its activity as evidenced in the hydrolysis of a specific substrate under defined conditions. Such determinations do not allow conclusions to be drawn about the activity of the enzyme in the cell. Yet barring intracellular changes in the ratio of enzyme to inhibitor or activator, measurements showing increase in enzyme activity are *prima facie* evidence of enzyme synthesis.

The present data, showing peptidase synthesis in cells whose growth is uncomplicated by mitosis, complement the earlier work on other organisms. In the histochemical analysis of the barley root (Linderstrøm-Lang and Holter, 1932; Bottelier, Holter, and Linderstrøm-Lang, 1943) an attempt was made to distinguish between the possible rôles of the peptidases in cell division and in growth. In the root tip, maximum enzyme activity per unit mass appeared in the zone, not of maximum mitotic rate but in the region just beyond, where total nitrogen per unit mass is maximal and where presumably increase in "active propoplasm" is greatest. It was this correlation which led Linderstrøm-Lang and Holter (1932) to suggest that peptidase content is high in regions of active intracellular synthesis. This thesis received support from the later work of Levy and Palmer (1940 *b*) who showed a parallelism between increase in total nitrogen and AG-peptidase in the chick, as well as from that of Pickford (1943), who correlated AG-peptidase content with the extractable protein in the salamander embryo. Similarly, Dumm (1943) found a peak of peptidase content corresponding to the period of rapid growth of the fetal liver in the rat. In all these cases, the intermixture of dividing and non-dividing cells and the presence of cells at different stages of division blurred the picture.

Attempts to achieve greater precision, carried out by Linderstrøm-Lang, Holter, and their group, took two directions. One was the analysis of the cytological structures to which the AG-peptidase might be bound. The peptidase activity of the cells studied was not bound to any of the larger granules but seemed distributed uniformly throughout the "active protoplasm" (Holter, 1936; Holter and Kopac, 1937). Thus the attempt to study peptidase activity by localizing its function gave a result which, while consistent with the idea that peptidases have to do with synthesis, did not help to define their rôle.

The second approach was a classic series of experiments designed to analyze the changes in enzyme distribution during early cleavage in marine eggs, where possible relations to the processes of differentiation could be studied (Doyle, 1938; Holter, Lanz, and Linderstrøm-Lang, 1938; Holter and Lindahl, 1941). Again the AG-peptidase was distributed in proportion to the "active protoplasm" and again no association with any specific function or structure appeared. Of major importance for the present discussion, however, is the finding that no increase in peptidase content occurred during the early cleavages. This period of development is precisely the time during which no increase in

nitrogen occurs (Ephrussi and Rapkine, 1928) and is an example of a biological system in which reproduction of the chromosomes takes place at the expense of components already present in the egg; *i.e.*, no new cytoplasm is formed. Thus, in the sea urchin egg, the formation of chromosomes need not be accompanied by increase in peptidase content.

The sea urchin data are reinforced by the findings of Doyle and Patterson (1942) on the protozoon *Didinium*. Two divisions after feeding, the total content of AG-peptidase in the daughter cells remained equal to that of the original parent. Thus, in these cases, division is accomplished without increase of either total mass or peptidase content. Conversely, the present studies of the salivary gland show that an increase of cell size without division is accompanied by an increase in the enzyme content. A consistent pattern appears: the association of AG-peptidase with the processes of synthesis in the cell.

The work of Duspiva (1942) on AG-peptidase during the growth of the frog oocyte provides an important complement to the argument. Here is still another type of cell, one in which cytoplasm is being formed to support the subsequent rapid embryonic cleavages. In this material the various phases of growth are seasonal and can be separated. Measuring the peptidase content per unit cytoplasm, Duspiva found a rise in concentration at the onset of yolk synthesis, then a maximum, with a later decrease explained by "dilution" of the cytoplasm by inert yolk. A recalculation of his data can be made in terms of the total peptidase per cell. On this basis, the increase in peptidase content continues to the end of the growth period as does the synthesis of yolk. In this case, as in the case of the salivary gland, the increase in enzyme is correlated with other syntheses in the cell.

The concept of an "active protoplasm," of which the peptidase content is characteristic, was used by Linderstrøm-Lang and Holter to interpret their data. On closer examination, this implies that the enzyme increases together with the functional working of the cell. Perhaps the simplest hypothesis is that the peptidase is part of a system of coupled reactions forming a unit of protein synthesis in the cell.

This suggestion, that AG-peptidase forms part of a biological unit of synthesis, requires examination in terms of other components of such a hypothetical system. The nucleoproteins deserve first consideration in view of the suggestions that they have to do with protein synthesis (see Caspersson, 1947; Brachet, 1947). A series of analyses of ultraviolet absorption spectra of cells of the salivary gland at different stages in the larval period (Schultz and Caspersson, 1938) is relevant to this question. The changes in the spectra could be interpreted as being due to a decrease in the pentosenucleic acid (PNA) to protein ratio of the salivary gland cytoplasm as the cell approached maturity. The differentiation of the cell from its embryonic state entails a decrease in

the nucleic acid concentration, even though the total amount increases. It is not possible as yet to correlate the peptidase data with the nucleic acid information, since different stages of the life cycle of the gland are covered by the two sets of data. It is essential to establish a precise correlation of the peptidase with the nucleoprotein data, of the sort carried out by Thorell (1947) for nucleic acid and hemoglobin synthesis in the blood cell.

In the plant root there is some evidence of a correlation between high PNA in the cytoplasm and high peptidase content. The data of Bottelier, Holter, and Linderstrøm-Lang (1943) show, for the barley root, a region of maximum peptidase content in the young cells, and a gradual fall in the older cells of the root. It is well known that the basophilic character of the cytoplasm decreases in the older cells, thus paralleling the decrease in peptidase content. For the onion root, the ultraviolet absorption data (Caspersson and Schultz, 1939) show a change from a high cytoplasmic nucleic acid concentration in the young cells to a very low concentration in those cells that have reached their complete differentiation.

Additional correlations between growth, peptidase content, and cytoplasmic PNA content can be found in other materials. In the sea urchin, according to Schmidt *et al.* (1948), the PNA content is constant during early development—the same period as that in which the peptidase is constant according to the Carlsberg group. Conversely, in such cases as the chick embryo and the rat liver, where the AG-peptidase content increases (see above) the PNA content increases (Caspersson and Thorell, 1941; Davidson, 1947; Novikoff and Potter, 1948). Such correlations may, of course, mean nothing more than the platitude that the growth of a cell involves the increase of its parts. Consideration of the behavior of other systems should provide a means of distinguishing real from spurious associations in growth processes. The concept, therefore, of a unit of synthesis involving the nucleic acids and a group of enzymes, including the peptidases, may prove useful in orienting further experiments particularly in connection with the stimulating hypothesis of Brachet and Chantrenne (1944) that the granules of the cytoplasm themselves may have a developmental history. On such a basis the apparent uniform distribution in cytoplasm of AG-peptidase and other peptidases may only mean an association with the smallest of the granules, those which in the Brachet and Chantrenne hypothesis are considered to be the progenitors of the larger and more elaborate ones.

The contrasting behavior of the AG-peptidase and the endopeptidase studied deserves some comment. The appearance of activity towards BAA in the histolyzing salivary gland suggests that the endopeptidase may function in the process of cell breakdown. The finding of Zamecnik and Stephenson (1947) can be interpreted in this light. They found appreciably higher activity towards BAA in hepatoma than in the control adult and fetal liver although the dipep-

tidase activities were comparable in the three tissues. In the tumors there is not only the possibility that necrotic tissue may have been included, but there is also present the type B cell of Caspersson and Santesson (1942), which is at a stage along the road to breakdown. It is possible that the endopeptidases begin to function at a time when protein denaturation has exposed susceptible peptide linkages.

The preceding discussion, biological in nature, has left untouched the question of the actual mechanism by which the peptidases may be related to protein synthesis. The recent discussions of this problem, those of Fruton excepted, have tended to minimize the possibility that the synthesis of peptide linkages is mediated by peptidases. The preferred view looks to phosphorylations to provide the requisite energy. It should be pointed out, however, that the peptidases may play a rôle of importance simply by carrying out their hydrolytic functions. The AG-peptidases (and also the other exopeptidases) may have the function of permitting amino acid transport from protein to protein, as members of a series of coupled reactions, thus playing a part in the exchanges shown to occur by the work with isotopes (Schoenheimer and Rittenberg, 1940). In this way they would permit the mobilization of specific amino acids at the particular places in the cell where the presence of energy-carrying phosphate groups allows synthesis to proceed.

SUMMARY

1. The larval salivary gland of *Drosophila melanogaster* offers an opportunity to study growth in a tissue in which no cell division occurs but in which the cells increase in size.

2. Measurements of alanylglycine (AG)-peptidase content have been made in three stocks of *Drosophila melanogaster* at different growth stages of the larval salivary gland, and have been correlated with its total nitrogen and volume.

3. During the prepupal instar, the AG-peptidase content of the gland increases parallel with total nitrogen but decreases when histolysis of the gland begins. Conversely, a benzoyl-*l*-arginineamide–hydrolyzing endopeptidase is not measurable until histolysis sets in.

4. In the final larval growth period of a giant mutant, there is a concomitant increase in peptidase, total nitrogen, and volume of the gland.

5. A similar association of peptidase content and total nitrogen is found in comparing glands of different sizes from the giant stock, at the time of maximal peptidase content in the prepupa.

6. The data are interpreted as evidence for an association of AG-peptidase with growth of the cells in the gland. This agrees with the earlier interpretation by Linderstrøm-Lang and Holter of data obtained from study of more complex tissues.

7. A survey of the available measurements of peptidase content in other

organisms shows that wherever an increase of cell substance occurs, peptidase content increases. Conversely, peptidase remains constant where cell division is unaccompanied by an increase of cell substances.

8. The joint association of peptidases and pentosenucleic acids with protein synthesis is pointed out.

9. The possiblity is considered that peptidases may be essential parts of a unit in which coupled reactions necessary for protein synthesis occur. The rôle of the peptidases in this system is discussed. They may act either synthetically to form new peptide linkages (problematic), or hydrolytically to mobilize the necessary specific amino acids.

Thanks are due our colleagues, Dr. R. W. Briggs, Dr. P. W. White, and Dr. J. A. Stekol for stimulating criticism during the preparation of the manuscript.

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